ULTRASTRUCTURAL OBSERVATIONS ON HOST-PARASITE RELATIONSHIPS OF THEILERIA ANNULATA AND THEILERIA PARVA IN VITRO

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DEDICATION

To my parents for their inestimable contribution to my education, and to my wife, Pamela, and sons Reg, Quincy and Archy, for their enduring patience and exemplary love.

DECLARATION

This thesis has been composed and written by me,

and describes my work.

CONTENTS

SUMMARY

Host-parasite interactions of *Theileria annulata* and *T. parva* *in vitro* have been investigated in this thesis.

An *in situ* ultrastructural study of sporogenic development of *T. annulata* in whole salivary glands from infected, 3-day fed *H. anatolicum anatolicum* ticks is described. It depicts sporogony as involving continuous growth and differentiation of a single sporont syncytium and appears to suggest that the original ramifying parasite mass eventually gives rise to smaller units, with one or more nuclei, from which sporozoites bud off by schizogony. *T. annulata* sporozoites measure an average of 0.9 μm long, 0.8 μm broad and possess a limiting unit membrane; an ovoid, non-chromocentric and anucleolate nucleus; double-membraned, acristate mitochondria; varying numbers of rhoptries, which together with the polar ring, form the apical complex; and numerous, loosely scattered ribosomal particles.

Penetration of lymphocytes by *T. annulata* sporozoites, as observed by light and electron microscopy in an *in vitro* culture system, is achieved as early as five minutes of incubation. The sporozoites invade target bovine lymphoid cells base first, the orientation which is dependent on the presence of receptors on the target cell plasmalemma and complementary recognition sites on the basal aspect of the parasite. The receptors have been shown to be susceptible to lysis by trypsin, and are most likely glycoproteins. The invasion of lymphoid cells and the interiorisation of *T. annulata* sporozoites is actively achieved by the parasite and is an

energy-dependent process which is markedly influenced by temperature. The sporozoites are endowed with intact functional metabolic energy pathways, and thus independently generate the ATP required for their invasive activities culminating in intracellular localisation. T. annulata sporozoites interiorise by a deepening invagination of the host lymphocyte plasmalemma which remains intact throughout the entry process and only fragments when the parasite is intracellular. T. annulata sporozoites form pellicular projections. It is believed that such localised pellicular distortions, following a firm parasite-lymphocyte membrane attachment, during active entry by a sporozoite could result in the invagination of host lymphocyte plasma membrane. The interiorised sporozoites, while they dedifferentiate into trophozoites, feed and transform into schizonts, are concomitantly subjected to host lysosomal activity. Viable developing trophozoites do not fuse with lysosomal vesicles, thereby circumventing enzymatic digestion. Failure of lysosomal fusion is attributed to structural alterations accompanying the transformation of interiorised T. annulata sporozoites into trophozoites.

Developing intracellular T. annulata and T. parva parasites provoke blastoid transformation of host lymphoid cells by insinuating gene fractions of their DNA. The nuclear envelope-derived annulate lamellae are believed to be a transcription product representing a species of mRNA and function in reciprocal communication of cyclical events in the host nucleus to Theileria schizonts so that through their degradation and assimilation, schizonts are able to monitor host cell chromosomal changes. The influence of Theileria on host

replicative machinery is illustrated in a lymphosarcomatous cell line (BL-20) superinfected by T. annulata where the parasite appears to take over control of the host cell replicative mechanism.

Light microscope autoradiography using tritiated thymidine demonstrated that Theileria schizonts synthesise their DNA during the G2 phase of host cell interphase stage and replicate their nuclei during the prometaphase stage of host cell cycle. The transformed cells, although susceptible to superinfection by a homologous Theileria species, cannot support the establishment of such interiorised sporozoites as schizonts. The interference is believed to be achieved by the incumbent schizont by blocking host cell gene sites.

CHAPTER ONE

INTRODUCTION

The animal disease complex, theileriosis, is caused by the obligately intracellular protozoan parasites in the genus Theileria. They are heteroxenous, undergoing a cyclical development a portion of which is spent in an appropriate ixodid tick and part in a bovid host. Cattle pathogens, Theileria annulata and Theileria parva, are transmitted by ticks belonging to the genera Hyalomma and Rhipicephalus respectively. In the tick, the host in which the sexual stage of Theileria species is believed to occur, the parasites develop transstadially. A few days after the immature stages of the tick vector have fed on, and dropped off, an infected animal in the overt stage of the disease, "male" (microgamonts and microgametes) and "female" (macrogametes) forms of Theileria are detectable in the intestine of the tick. Consequent on syngamy, the two forms give rise to ovoid zygotes which enter the lining epithelial cells of the tick gut and transform into kinetes. As the tick moults to the next stage in its life cycle the kinetes migrate through the haemolymph to the salivary glands, infect specific acinar cells and remain dormant until the tick feeds when intense development and multiplication culminate in the formation of large numbers of infective sporozoites.

The asexual phase of the parasite development is initiated in a susceptible vertebrate host when viable sporozoites, inoculated with saliva of an infected tick during feeding, reach and invade lymphoid cells in the local drainage lymph node. Several days after sporozoite inoculation, depending on the dose of infective particles

cells undergo blastoid transformation following which multinucleate schizonts appear in occasional lymphoblasts and can be detected in needle biopsies of the local drainage lymph node. Schizont-containing lymphoblasts undergo intense mitotic activity, rapidly proliferate and are disseminated systemically, thus provoking a severe febrile reaction in the host animal. During the replication of infected lymphoblastoid cells, the schizont situates itself in the mitotic plane of the dividing host cell and is cleaved into two halves so that the resultant daughter cells each contains a daughter schizont. After a certain number of such synchronized schizont-host lymphoblast proliferations, the parasite undergoes schizogonic multiplication and produces numerous uninucleate merozoites which invade host erythrocytes to become intraerythrocytic piroplasms, the development of which is time dependent and is not influenced by the dose of infective particles inoculated into a susceptible vertebrate host animal. Piroplasms are infective for ticks, and when ingested, readily initiate the sexual phase of the Theileria life cycle in susceptible tick species.

The above is a generalised description of the life cycle of Theileria species which is still incompletely defined. In the vertebrate host, the events which precede the appearance of the schizont stage in the local drainage lymph node of a freshly infected animal provoke major questions about: (1) mechanisms of sporozoite-host lymphocyte recognition, (2) sequential structural changes concomitant with the pre-schizontal development, (3) parasite metabolic pathways activated during sporozoite-lymphocyte interactions and (4) mechanisms of intracellular survival. How Theileria schizonts cause blastoid

transformation of their host cells and how they regulate and monitor cellular events to achieve synchrony between schizont cleavage and host cell division are other challenging questions.

This thesis is based on investigations pertinent to the above questions with particular reference to Theileria annulata and Theileria parva in vitro.

Two papers which constitute parts of the work included in this study were published prior to its submission and are included as appendices to the thesis.

CHAPTER TWO

REVIEW OF THE LITERATURE

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2.1 Taxonomy

Theileria Bettencourt, Franca and Borges, 1907 are Protozoa Leuckart, 1879. Owing to deficient structural descriptions and incomplete understanding of the various phases of their life cycle, their taxonomic standpoint is controversial. According to the traditional classification (Wenyon, 1926), they were placed in the class Sporozoa Leuckart, 1879 within the subphylum Plasmodroma Doflein, 1901. The subphylum comprised forms which had either pseudopodia or flagella as organs of locomotion, and parasitic Sporozoa which, owing to their mode of life, had been modified in various ways so that there was either a single or more than one vesicular nucleus present. Sexual reproduction was considered to take place either by anisogamy or isogamy. Many forms were heterogenetic, asexual and sexual modes of reproduction alternating with successive generations. Members of the class Sporozoa reproduced characteristically by schizogony. Levine (1961), however, recognised lack of affinity of piroplasms, the Babesiidae Poche, 1913 and the Theileriidae du Toit, 1918, with typical Sporozoans such as Haemosporina Danilewsky, 1885. The uncertainty regarding the existence of sexual reproduction in the life cycle of piroplasms led him to suggest the creation of a class Piroplasmea Wenyon, 1926 as an appendage to the Protozoa. A Committee of the Society of Protozoologists (Honigberg et al., 1964) not only found the former position of piroplasms among the Sporozoa doubtful but also recognised that their relationship to the Sarcodina was quite clear. The Committee

consequently transferred Piroplasmea to the superclass Sarcodina Hertwig and Lesser, 1874 in the subphylum Sarcomastigophora Honigberg and Balamuth, 1963. Most members of the superclass are free-living and typically possess pseudopodia. Flagella, when present, are restricted to developmental stages. Their bodies are either naked or have external or internal skeletons of various chemical composition. Asexual reproduction is by fission while sexual reproduction, if present, involves flagellate or, more rarely, amoeboid gametes. The piroplasms, however, did not last long among the Sarcodina. Ultrastructural characteristics, pertinent mainly to the specialised complex of organelles in their apical region, soon prompted Levine (1969, 1971, 1973) to, once more, transfer the Theileriidae and the Babesiidae back into the sporozoan fold in a separate class Piroplasmasida which together with the class Sporozoasida constituted the subphylum Apicomplexa Levine, 1970. The Theileriidae had a much reduced apical complex consisting only of rhoptries unlike the Babesiidae which had, in addition to the rhoptries, not only a polar ring and subpellicular tubules but also a conoid in some species. In the meantime a great deal of new taxonomic information was accumulating, much of it acquired through electron microscopy, especially on the life cycle of the Piroplasmasida in vector ticks. It was becoming increasingly evident that they were more closely related to the Haemosporina. In a newly revised classification of the Protozoa (Levine et al., 1980) the classes Piroplasmasida and Sporozoasida were, therefore, once again merged in a new class Sporozoea in the phylum Apicomplexa, the subkingdom Protozoa and the kingdom Protista Haeckel, 1866. The nomenclatural terminology used in this thesis is

based on the above newly revised classification (Levine et al., 1980).

The question of the validity of genera within the family Theileriidae du Toit, 1918 has, similar to the higher taxa of piroplasms, been a subject of controversy. Traditionally all the non-pigmented blood parasites of animals were called Piroplasma Patton, 1895. The aetiological agent of East Coast fever, first recognised by Koch (1898) in cattle suffering from redwater and thought to be young forms of Babesia (Piroplasma) bigeminum Smith and Kilborne, 1893, was initially named Piroplasma Kochi by Stephens and Christophers (1903) and later Piroplasma parvum by Theiler (1904). Dschunkowsky and Luhs (1903) described a highly fatal disease, tropical piroplasmosis, in Transcaucasian cattle, the cause of which resembled, but was not identical with, Piroplasma parvum. It was named Piroplasma annulatum (Dschunkowsky and Luhs, 1904). Another intraerythrocytic parasite which produced an extremely mild cattle disease was described and named Piroplasma mutans (Theiler, 1906). Bettencourt, Franca and Borges (1907) compared the life cycle of Piroplasma bigeminum with those of Piroplasma parvum and Piroplasma annulatum and were convinced that the presence of schizogony in the life-cycles of the latter two piroplasms justified their removal from the genus Piroplasma. A genus Theileria Bettencourt, Franca and Borges, 1907 was thus created so that Piroplasma parvum, P. annulatum and P. mutans became Theileria parva (Theiler, 1904), T. annulata (Dschunkowsky and Luhs, 1904) and T. mutans (Theiler, 1906) respectively. The description of other species soon followed. Littlewood (1915) and Mason (1915) encountered a highly pathogenic piroplasm in Egyptian and

Sudanese sheep. It was distinct from Babesia ovis. Mason (1915) placed this parasite in the genus Theileria without giving it a specific name. Rodhain (1916) encountered another piroplasm in apparently healthy sheep in the Belgian Congo (Zaire) and named it Theileria ovis. Du Toit (1918) created a new family Theileriidae and placed in it those Theileria species which not only multiplied by schizogony within lymphocytes but also by binary fission in erythrocytes. The species assigned to the new family included Theileria parva, T. annulata and the unspeciated, highly pathogenic Theileria parasite described in Egyptian sheep (Mason, 1915). Du Toit (1918) named this parasite Theileria ovis Littlewood, 1915. He transferred Theileria mutans, which had not manifested schizogony in its development, to a new genus Gonderia du Toit, 1918 in the family Babesiidae. Sergent, Parrot and Hilbert (1922) transferred the non-pathogenic Theileria ovis Rodhain, 1916 described in healthy sheep in the Belgian Congo (Rodhain, 1916) to the genus Gonderia due to an apparent lack of schizonts in its life-cycle and named it Gonderia ovis. Wenyon (1926) changed the name of the pathogenic Theileria ovis Littlewood, 1915 to Theileria hirci Dschunkowsky and Urodschevich, 1924 because the specific name ovis had already been used for the non-pathogenic counterpart Theileria ovis Rodhain 1916. In the same year Wenyon (1926) banished all the previous Theileria species in which schizogony had not been proved to the family Babesiidae. To this family he transferred Gonderia mutans and G. ovis which consequently became Babesia mutans and B. ovis. A new name, Babesia sergenti, was proposed for the latter piroplasm by Wenyon (1926). He, however, retained Theileria parva, T. annulata and T. hirci in

the family Theileriidae. Doyle (1924) and Theiler and Graf (1928) demonstrated schizogony in the life-cycle of Babesia mutans. It was, therefore, once again transferred to the genus Theileria by Theiler and Graf (1928). Around the same time Lestoquard (1929) demonstrated schizonts in sheep and goats infected with Babesia ovis, introduced the parasite into the genus Theileria and renamed it Theileria recondita. After reviewing the classification of piroplasms, Thomson and Hall (1933) recommended that the non-pathogenic Theileria species of sheep and goats be called Theileria ovis Rodhain, 1916. Lawrence (1933, 1934) in Southern Rhodesia (Zimbabwe) Neitz, Adelaar and Kluge (1953) and Neitz, Canham and Kluge (1955) in the Corridor between Hluhluwe and Umfolozi Game Reserves in Zululand, South Africa, described a cattle disease which caused high mortality and whose epidemiological pattern was unique. Neitz, Canham and Kluge (1955) observed that the tick, Rhipicephalus appendiculatus, was the invertebrate host. Neitz (1955) not only observed that the African buffalo (Syncerus caffer) served as a reservoir of infection for ticks but also that the causative parasite was distinct from either Theileria parva or T. mutans. He named it Gonderia lawrencei. Another piroplasm, Cytauxzoon sylvicaprae, was described by Neitz and Thomas (1948) in a duiker (Sylvicapra grimmia). The erythrocytic forms resembled T. mutans but the schizonts were larger than those of other known Theileria species and were detectable in uni- and multinucleate histiocytes. Neitz and Jansen (1956) reclassified the Theileriidae on the basis of the phases of the life-cycle in which multiplication took place. They redefined and reinstated the generic name Gonderia to include piroplasms which multiplied

by schizogony in lymphocytes and later by binary fission in erythrocytes. They transferred the parasites in this category, together with the genus Cytauxzoon which underwent schizogony in histiocytes and binary fission in erythrocytes, to the family Gonderiidae Neitz and Jansen, 1955. Theileria annulata, T. hirci, T. mutans and T. ovis thus became Gonderia annulata, G. hirci, G. mutans and G. ovis respectively. Neitz and Jansen (1956) also included Gonderia lawrencei in the family. They retained Theileria parva, which only multiplied by schizogony in lymphocytes and did not divide in erythrocytes, as the only genus and species in the family Theileriidae. In 1956, Neitz and De Lange described a new parasite, Cytauxzoon strepsicerosi, in a kudu. Another species, Cytauxzoon taurotragi, was described in an eland (Taurotragus oryx) by Martin and Brocklesby (1960). Neitz (1962) abolished the family Gonderiidae and the genus Gonderia when he believed he had demonstrated multiplication of erythrocytic forms of Theileria parva in splenectomized calves in the complete absence of schizonts. The family Theileriidae now comprised two genera, Theileria and Cytauxzoon. Barnett (1968) included the cattle parasite, Hematoxenus veliferus Uilenberg, 1964 in the family Theileriidae thus increasing the number of genera in this family to three. Levine (1971) synonymized the genus Cytauxzoon with Theileria, leaving two genera, Theileria and Hemotoxenus in the family. Brocklesby (1978) did not, however, share the same view and retained Cytauxzoon as a genus within the family Theileriidae. Around the same time, Van Vorstenbosch et al. (1978) abolished the genus Hematoxenus and synonymized it with Theileria on the basis of ultrastructural similarities between the two genera. Later work

(Grootenhuis et al., 1979) demonstrated that Cytauxzoon taurotragi infected and developed in cattle in a similar manner to Theileria parasites. They consequently synonymized Cytauxzoon with Theileria and renamed the parasite Theileria taurotragi Martin and Brocklesby (1960) thus leaving only one genus, Theileria, within the family Theileriidae.

2.2 Distribution

2.2.1 East Coast fever (ECF)

The distribution of ECF (Theileria parva infection) is generally coincident with the areas ecologically suitable for the occurrence of the three-host arthropod vector, the ixodid tick, Rhipicephalus appendiculatus (Daubney, 1938) so that the disease is most prevalent where the tick is most abundant. R. appendiculatus is restricted to Africa south of the Sahara, in East, Central and South Africa (Barnett, 1968). Although it requires a warm, relatively humid, bushy grass country and occurs in areas with annual rainfall of about 15 inches (Theiler, 1949), it is not found in the tropical rain forests of Central and Western Africa. It is most frequently found in country between about 4,000 and 7,000 feet in altitude with adequate vegetation cover and a well-distributed rainfall of about 25 inches per year or more (Walker, 1970) but is capable of living in places with a lower annual rainfall, provided the dry season is not too long, or at higher or lower altitudes. The soils of a red-chocolate loam formation carry vegetation suitable for R. appendiculatus and it is very difficult to eradicate ECF from such areas (Daubney and Hudson, 1931). Tick collection

and identification and the indirect fluorescent antibody (IFA) test (Morzaria et al., 1981) have, however, not only demonstrated the occurrence of R. appendiculatus in the dry, excessively hot (30°C) Juba and Aswa River areas of southern Sudan, but have also confirmed the overlapping of T. parva and T. annulata infections in that region. The ability of R. appendiculatus to tolerate daily temperature maxima in excess of 30°C or dry seasons of more than four months in Sudan is of particular interest especially in the light of the observation that exposing R. appendiculatus nymphs or adults to continuous, high temperatures (31 to 38°C) leads to a loss of virulence by T. parva (Lewis, 1950). In East Africa R. appendiculatus is found primarily in the ecological zone encompassing the humid to dry, sub-humid areas (Pratt, Greenway and Gwynne, 1966). It is distributed in the highlands of Kenya and Tanzania; along a broad belt stretching 25 to 50 miles away from the shores of Lake Victoria in Kenya, Uganda and Tanzania and continues to be found down the Rift Valley in the country adjacent to Lake Tanganyika and Lake Nyasa (Lake Malawi) and in Mozambique (Wilson, 1953). The tick is also found along the wet coastal belt of Tanzania and Kenya, stretching only a few miles inland in Kenya, but much farther inland in Tanzania. Yeoman (1966) described an ECF-enzootic area in Sukumaland, Tanzania. He observed that the heavier, better seasonally-distributed rainfall (30-40 inches) on the Lake shore (October-May) with its high humidity (mean annual vapour pressure about 20 millibars), a perennial tall grass complex, and a mean annual maximum temperature of 27-30°C was clearly suitable for R. appendiculatus. Similar conditions were reported in Uganda (Oteng, 1970) where ECF-enzootic areas receive rainfall of at least

40 inches annually, well-distributed throughout the year with one broad peak in the north and a double peak in the south. The savanna type vegetation in these areas is dominated by the tall Hyperenia grass species in the north and the elephant grass, Pennisetum purperus, in the south.

In addition to the main vector, R. appendiculatus, other tick species of this genus and ticks belonging to other genera have been incriminated in the transmission of T. parva infection, mainly under experimental conditions. R. evertsi (Lounsbury, 1906; Fotheringham and Lewis, 1937) has been incriminated. This tick, together with R. appendiculatus, has been reported at an altitude of 5,000 feet above sea level in South Africa (Theiler, 1950) and at 6,000 to 7,000 feet in Kenya (Lewis, 1950). Lewis, Piercy and Wiley (1946) reported R. neavei as a vector of T. parva. Neitz (1957) observed that the distribution of R. neavei in Kenya frequently overlapped with that of R. appendiculatus and extended widely into areas where the latter tick was apparently unable to survive so that in the absence of other Rhipicephalus species, R. neavei could maintain ECF. Wilson (1953) reported the discovery of two new tick vectors of T. parva, R. jeanelli and R. ayrei. Both of these ticks occur within the R. appendiculatus zone but their distribution within this zone is limited to upland forest areas. Using nymphs of R. pulchellus isolated from Beisa oryx (Oryx beisa beisa) on El Barta plains near Baragoi, Kenya, and allowed to engorge on four cattle infected with T. parva (Muguga), Brocklesby (1965) succeeded in transmitting one typical case of ECF. R. carnivoralis had been shown to be capable of transmitting T. parva under experimental

conditions (Brocklesby, Bailey and Vidler, 1966) and stages of the protozoan had been found in thin sections of the salivary glands of the tick.

Certain Hyalomma species have also been implicated in the transmission of ECF. Fotheringham and Lewis (1936) reported Hyalomma dromedarii and H. truncatum while Lewis and Fotheringham (1941) observed that H. excavatum could transmit the disease. Neitz (1957), while admitting that the three Hyalomma species had been proved transmitters of T. parva infection under experimental conditions, emphasised that there was no evidence indicating natural transmission of ECF by Hyalomma species. More recently Mehlhorn and Schein (1976) have demonstrated ultrastructurally that T. parva can develop within the intestine of Hyalomma anatolicum excavatum, at least under experimental conditions. They observed that on the 2nd-4th days following repletion, the intestine of the ticks contained numerous stages which were very similar to microgamonts and microgametes of haemosporidia.

Amblyomma variegatum was incriminated by Uilenberg, Robson and Pedersen (1974) as a vector of T. parva but the doubt cast on the validity of the results is reflected in the authors' own words as they summarised "Amblyomma variegatum may have transmitted Theileria parva on one occasion but circumstantial evidence makes this observation doubtful". Earlier attempts by Fotheringham and Lewis (1937) to transmit T. parva by A. variegatum were not successful.

The African buffalo (Syncerus caffer) is not only susceptible to infection by T. parva (Lewis, 1943; Barnett and Brocklesby, 1966) but is also capable of harbouring it, thus acting as a reservoir,

efficiently maintaining the infection in the absence of cattle. The source and maintenance of infection in Rhipicephalus species may either be fully susceptible cattle and buffaloes reacting to ECF or partially immune cattle and possibly also African buffaloes that develop a microscopic infection after reinfestation with infected ticks (Neitz, 1957). The occurrence and dissemination of ECF are favoured by the availability of a dense cattle population, partial or complete absence of fences, uncontrolled movement of livestock within an enzootic area or into a potential ECF area and a high incidence of vectors (Neitz, 1957).

2.2.2 Tropical theileriosis

Tropical theileriosis (Theileria annulata infection) is a widespread protozoan disease of cattle in northern tropical, subtropical, Mediterranean and temperate countries in which the ixodid vector ticks of the genus Hyalomma are present. H. detritum, considered the most efficient vector (Barnett, 1968) because immature stages feed readily on cattle, has been observed to be responsible for transmitting the disease in North Africa (Sergent, Donatien, Parrot and Lestoquard, 1928) and in Russia (Tselishcheva, 1940). Ticks of the H. anatolicum complex occur from North-west Africa through the Middle East and southern U.S.S.R. to India and Pakistan (Barnett, 1968) and have been incriminated in transmitting tropical theileriosis in this region (Delpy, 1949). The immature stages of H. anatolicum anatolicum feed on most animals from the size of a rodent upwards, and larvae and nymphs can acquire infection by feeding on cattle and transmit the disease after moulting when they feed as nymphs or adults (Barnett, 1977). H. anatolicum excavatum, found

over the same range and habitat as H. anatolicum anatolicum, has been shown to be widespread on cattle in Israel (Feldman-Muhsam and Saturen, 1961) and is involved in the transmission of T. annulata infection (Daubney and Said, 1951). Barnett (1977) was, however, not convinced that H. anatolicum excavatum transmits tropical theileriosis under natural conditions. He observed that the larvae and nymphs feed mainly, if not exclusively, on rodents, and that although the adult ticks feed on cattle and other large animals, cattle will not be infected because nymphs cannot acquire T. annulata from rodents. The tick will, on the other hand, readily transmit T. annulata experimentally (Hadani, Tsur, Pipano and Senft, 1963). H. dromedarii asiaticum (= H. asiaticum) was reported to be involved in the biological transmission of T. annulata in Central Asia (Tselishcheva, 1940). H. marginatum has been held responsible for transmitting T. annulata infection in Italy (Batteli, Memola and Pepe, 1954; Pieresca, 1958), in Yugoslavia (Angelovski and Iliev, 1977) and over the same range and habitat as the H. anatolicum complex (Delpy, 1949). H. detritum (Sergent, Donatien and Lestoquard, 1931) and H. anatolicum complex (Daubney and Said, 1951) are prone to hide in crevices, and have often been found in stone walls of stables where they remain dormant in winter. At the beginning of summer, the eggs hatch and the engorged larvae and/or nymphs moult, emerge from their hiding places when the weather conditions are favourable and attack cattle and other animals so that during this season and early autumn, a large number of susceptible animals contract T. annulata infection (Neitz, 1957). Apart from cattle, the other members of the family Bovidae susceptible to, and involved

in the distribution of, T. annulata infection include the Indian water buffalo, Bubalus bubalis, (Mason, 1922) and the Tibetan yak (Barnett, 1977). Naturally recovered animals develop an immunity which may wane within three years after recovery (Sturman, 1935). Although it is usually difficult to demonstrate T. annulata in immune animals, they serve as reservoirs of infection for ticks (Neitz, 1957) and splenectomy of such animals or their superinfection with Babesia or Anaplasma species is invariably followed by a relapse within three weeks (Sergent, Donatien, Parrot and Lestoquard, 1945). The distribution and prevalence of tropical theileriosis is, therefore, greatly influenced by the presence and density of transmitting Hyalomma ticks, the availability of susceptible or immune cattle or other Bovinae, the incidence of immunosuppressive hemoprotozoal or viral diseases such as babesiosis and/or anaplasmosis or foot and mouth disease, and uncontrolled movement of livestock within an enzootic area or into a potential tropical theileriosis region.

2.3 Life cycles of Theileria parva and Theileria annulata

2.3.1 In the naturally-transmitting tick

2.3.1(a) Theileria parva: Rhipicephalus appendiculatus, the tick responsible for transmitting T. parva infection in nature, requires three hosts to complete its life cycle (Walker, 1970). The larvae which hatch from eggs laid on grass by a replete adult female R. appendiculatus attach to, and feed on, a host animal, frequently bovine. When engorged, they fall to the ground, undergo ecdysis and develop into nymphs. These attach on a fresh host, feed and

fall off to moult into adult ticks which again seek a new host on which they feed and mate. Engorged adult R. appendiculatus ticks fall to the ground, the females lay eggs and a fresh cycle is initiated once more. The cycle of development of T. parva, like that of other Theileria species, is closely associated with that of its vector tick which transmits the disease transstadially (Gonder, 1910). If R. appendiculatus becomes infected as a larva, it can only transmit ECF after moulting to the nymphal stage. Similarly, if the tick acquires the infection as a nymph, it is only able to transmit the disease as an adult. The developmental cycle of T. parva in R. appendiculatus is initiated when susceptible larvae or nymphs feed on infected cattle or African buffalo, in the overt stage of ECF, in which the parasite has developed to the intra-erythrocytic piroplasm stage. Gonder (1911, 1911a) observed that within the tick gut, concomitant with a massive destruction of the ingested erythrocytes, there was emigration of parasites within the first 30 minutes, a great many of which perished; mature gametocytes developed into gametes and fused with each other; the fertilized microgametes became round and transformed into ookinetes which moved by doubling back and stretching out rapidly. In addition to these early forms Gonder (1911) described clusters of sporozoites in ticks already moulted after they fed on infected cattle which were ready for another feed on a fresh host. Although he suggested that the sporozoites must have come from the ookinetes, he neither demonstrated transitional stages nor did he describe the tick cells associated with the various developmental forms of T. parva. Cowdry and Ham (1932) examined smears, serial sections and dark field preparations

of infected nymphs each day after engorgement, through moulting and feeding to repletion on a susceptible animal. Like Gonder (1911), they observed the destruction of erythrocytes and emigration of T. parva forms in the gut of the ticks but noted that the process went on for a much longer period ranging from two to six days when no more intraerythrocytic parasites were detectable. Liberated parasites, mainly of two types were observed within the tick gut lumen. The large, roughly spherical form whose eccentric nuclei were in some cases constricted equatorially and partly divided into two parts, and the small uninucleate form with a relatively scanty cytoplasm which may be drawn out into a tail-like process. Cowdry and Ham (1932), while admitting that they had no supporting evidence, expressed the possibility that the free, large and small forms of T. parva which they had observed in the gut of R. appendiculatus nymphs could represent the female and male gametocytes respectively. The two dissimilar forms, observed for up to two weeks, became applied to the surface of the gut epithelial cells, occasionally in very close contact with each other. Certain epithelial cells took up many parasites, thus stretching further into the lumen than their neighbours in which only a small number of parasites was detected. The former group of cells was called "pedunculated" as opposed to their neighbours, referred to as "non-pedunculated", and were the cells in which further development of T. parva occurred. Once within the epithelial cells, both forms of the parasite underwent some degree of division, the small forms rapidly disappeared and a new form of T. parva, referred to as a zygote by Cowdry and Ham (1932) resulted. This stage possibly represented the round form, described by Gonder

(1911), which was produced by the fusion of gametocytes and which later transformed into ookinetes. The zygotes were initially larger and had single distinct nuclei and abundant, basophilic cytoplasm but later lost their spherical contours, became angular, almost spidery in shape with indistinct nuclei. From the day before moulting, about 15 days after the ingestion of blood containing T. parva piroplasms, the transformation of zygotes into ookinetes was depicted by the reappearance of distinct nuclei, ranging from one to four within individual zygotes, and the concentration of the substance of the zygote into a central mass representing the future ookinete. The limiting membrane of each ookinete, which appeared to develop in a kind of capsule formed by the transforming zygote, finally ruptured, the ookinete emigrated and entered the haemolymph of the tick.

Cowdry and Ham (1932) not only provided the above detail on the developmental stages of T. parva in the gut of R. appendiculatus nymphs but also the development of the ookinete within the salivary glands of the vector tick. The ookinetes entered and were detected within salivary gland cells as early as 23 days after engorgement (i.e. a day before moulting) to as late as 47 days after engorgement. The parasite developed into a sporont with peripheral buds, the sporoblasts, which underwent rapid development and differentiation culminating in the detachment and production of sporozoites. The production of sporozoites occurred on the third day after the infected nymphs had attached and continuously fed on susceptible cattle. Cowdry and Ham (1932), on the basis of this observation, believed and proposed a working hypothesis that the final development of T. parva in the tick, resulting in the parasite becoming infective, only

commenced after the tick had begun to take blood. This hypothesis was supported by the observations of Theiler and Du Toit (1928) and Walker (1930) that an infective adult tick transmitted ECF only after it had been biting for a period of not less than six hours.

Reichenow (1940) observed that when infected blood was ingested by the tick, the erythrocytes underwent rapid degeneration and liberated parasites, large numbers of which degenerated and were digested so that only a very small number survived. He reported that the free parasites, seldom seen in the gut three to five days following repletion, frequently appeared in large masses in various parts of the gut including the free surfaces of the pedunculated epithelial cells. Reichenow (1940) was convinced that the parasites which were taken in by the pedunculated cells were the ones which finally reached the granule-secreting alveoli of the salivary glands after passing through the haemolymph. This belief was, however, based on no evidence since he had failed to find the ookinetes. He was erroneously convinced that the developmental forms described by Cowdry and Ham (1932) in the gut such as the gametocytes and zygotes were artefacts. Reichenow (1940) was unable to detect any developmental stages of T. parva in the salivary glands until the tick had moulted and started feeding on a fresh host when he observed extensive multiplication. He observed, like Cowdry and Ham (1932), that the multiplication resulted in the production of minute unicellular parasites, resembling intraerythrocytic piroplasms, which appeared in very large numbers approximately three days after the infective R. appendiculatus had attached on a new host. He was convinced that T. parva multiplied by repeated binary fission.

The salivary glands of Rhipicephalus appendiculatus comprise various distinct types of alveoli or acini (Till, 1961). Three types are recognised in the female tick, one non-granular and two granular, designated as types I, II and III. In the male tick, there is a fourth, type IV, which is inconspicuous and agranular in the non-infected tick. Martin, Barnett and Vidler (1964), like Reichenow (1940), were not only unable to find parasites in the non-pedunculated epithelial cells of the tick gut but also saw no zygotes, ookinetes, or any suggestion of sexual stages that were observed by Cowdry and Ham (1932). Martin et al. (1964) erroneously asserted that the forms of T. parva described by Cowdry and Ham (1932) in the gut of R. appendiculatus nymphs represented nothing more than degenerate tick tissue cells. Like Reichenow (1940), Martin et al. (1964) could not demonstrate the parasite in salivary gland cells until the tick had moulted to the next instar and started feeding on a fresh host. They observed that only acini of types II and III played a role in the cyclic development of T. parva and that in these acini only the granule-secreting cells were parasitized. One or more cells of the same acinus were shown to be infected, and both immature and infective stages were frequently found in the same tick. They observed that parasite masses became progressively larger as development continued and eventually occupied the whole cell thus eliminating the secretory droplets. The parasite masses often developed into multinucleate clumps, the cytomeres, which produced sporozoites by multiple fission. Martin et al. (1964), like Cowdry and Ham (1932), reported maximum production of sporozoites in a moulted tick about four days after it had attached and continuously fed on a fresh host.

They, however, disagreed with the latter authors when they observed that mature, infective sporozoites were present in ticks as early as one day after they fed on a fresh host as opposed to three days.

The early descriptions of the developmental forms of T. parva in the gut of R. appendiculatus nymphs by Cowdry and Ham (1932) were later confirmed with greater detail by Schein, Warnecke and Kirmse (1977). The lysis of erythrocytes in the gut of nymphs led to the liberation of free piroplasms which asynchronously differentiated into male (microgametes) and female (macrogametes) forms of T. parva. The development of the male forms began 12 hours after repletion with the growing of a "spine" in the round forms which then stretched out to become spindle-shaped and which were considered to be macrogametes. The differentiation of microgamonts into microgametes began when the former adopted a polymorphous shape and developed flagellate cytoplasmic projections. Microgamete nuclei divided so that they became multinucleate. The forms considered to be macrogametes were characteristically round and were frequently seen clustered. Schein et al. (1977) were not certain whether the clustering resulted from the division or mere conglomeration of the macrogametes. They observed round forms of the parasite within epithelial cells of the tick gut which showed a definite increase in size four days after repletion and which differentiated into zygotes six days after the nymphs engorged on blood containing T. parva piroplasms. The timing of the development of zygotes is in general agreement with the observation of Cowdry and Ham (1932) that zygotes of T. parva were present in gut epithelial cells in different batches of nymphs over periods of 6-17, 9-34, 6-28 and 7-23 days after engorgement on an animal

suffering from ECF. Schein et al. (1977) reported the development of club-shaped kinetes from zygotes three to five days after the nymphs moulted to adult R. appendiculatus (i.e. 21-23 days following repletion). Like Cowdry and Ham (1932), Schein et al. (1977) were, however, unable to demonstrate syngamy of the micro- and macrogametes. The development of kinetes from zygotes was studied in greater detail by electron microscopy (Mehlhorn, Schein and Warnecke, 1978). They observed that two to six days after repletion the gut of R. appendiculatus nymphs contained numerous microgamonts and microgametes with ultrastructural characteristics described by Mehlhorn and Schein (1976). The microgamonts, which were multinucleate, measured about 10.5 µm with a maximum diameter of 2.1 µm in their middle region, had stiletto-like apices, several flagellate protrusions about 3 µm long, two slender posterior projections and were bounded by a unit membrane. The microgametes were more or less cord shaped, had a central thickening caused by the nucleus and several microtubules which ran from one end to the other. Mehlhorn et al. (1978) concurrently detected macrogametes which measured 4-5 µm maximum diameter, were ovoid, characterised by a relatively pale cytoplasm containing small vacuoles, double-walled structures and several electron-dense granules. After the nymphs moulted into adults, i.e. around 20 days after repletion, the zygotes, which measured 6-7 µm in diameter and which had single nuclei located at the margin and a large vacuole close to the nucleus, were detected. Kinete development started within the zygote when a portion of the cytoplasm of the latter protruded into the large adnuclear vacuole. The protrusion was limited by a vacuolar membrane under which two

newly formed membranes, adjacent to each other appeared. As the development continued, the large vacuole extended while the protrusion was considerably enlarged so that finally only a small zone of the cytoplasm surrounded the large vacuole containing the differentiating stage. The newly forming kinete remained connected with the residual body by a small cytoplasmic bridge. Finally it attained a club shape within the vacuole prior to the residual body being ruptured, thus liberating a motile parasite. The kinetes which became numerous three to five days after ticks moulted, elongated and started moving within the intestinal cells of the adult tick. In several kinetes nuclear division started so that parasites with up to four nuclei were observed. Around three days after tick moult, the kinetes, some of which were multinucleated emigrated from gut epithelial cells, were found within the haemolymph, and finally penetrated the salivary gland cells. Mehlhorn et al. (1978) failed to detect any development of kinetes before the ticks moulted. It is, however, noteworthy that Cowdry and Ham (1932) found kinetes as early as a day before ticks moulted. Of the different salivary gland acinar types in R. appendiculatus (Till, 1961), Martin et al. (1964) reported that T. parva developed in both types II and III. Fawcett, Buscher and Doxsey (1982), on the other hand, observed that only type III acinus was involved in the development of T. parva and that in this type, only the e-cell was susceptible to infection by the parasite. The e-cells occur only in type III acinus and have very large spherical secretory granules, 4-6 μm in diameter which give strong histochemical reactions for protein with bromphenol blue and for lipid with Sudan black, but are unreactive for

carbohydrate in the periodic acid-Schiff reaction (Binnington, 1978). Fawcett, Buscher and Doxsey (1982a) undertook an ultrastructural study of the sporogonic development of T. parva in the salivary glands of moulted R. appendiculatus ticks unfed and, fed on rabbits from 15 hours to eight days. They reported that the earliest stages of the parasite observed within the e-cells already possessed several irregular, pleomorphic nuclei thus corroborating the observation of Mehlhorn et al. (1978) that sporogony, as depicted by kinete nuclear division, commenced before the kinetes entered salivary gland cells. Fawcett et al. (1982a) proposed that sporogony of T. parva within e-cells involved a continuous growth and differentiation of an individual early stage, the sporont, a multinucleate syncytium of increasing three-dimensional complexity until it gave rise to sporozoites in a terminal episode of cytoplasmic fission. The authors, however, commented that their proposal was not easy to prove. They did not observe the cytomere stage described by Martin et al. (1964) and did not believe there were any secondary or tertiary stages before the final production of infective sporozoites. Fawcett et al. (1982a), therefore, believed that these stages possibly represented artefactitious fragmentation of a coherent mass of parasite protoplasm during such preparatory techniques as squashing the salivary gland acini for light microscopy.

2.3.1(b) Theileria annulata: The developmental stages of T. annulata were studied by Sergent, Donatien, Parrot and Lestoquard (1936) in the two-host tick, Hyalomma mauretanicum (= H. detritum). They observed that during the early days following ingestion of T. annulata-infected blood by larvae and nymphs, the gametocytes promptly

emigrated from the erythrocytes and agglomerated in masses in the intestinal lumen. The majority of the liberated parasites degenerated in the intestinal lumen. They not only found it impossible to confidently distinguish the macrogametes and microgametes but were also not certain about the occurrence of fertilization. Sergent et al. (1936), however, believed that the process of syngamy took place within the intestine of H. detritum 13-21 days after they fed on an infected animal. During this period, young zygotes, spherical or irregularly polygonal to cuboidal, were detected within the non-pedunculated and pedunculated gut epithelial cells. Around the third week after the repletion of the nymphs, the zygotes became encysted and remained in that state during the six months of hibernation in winter up to the time the nymphs moulted. According to Sergent et al. (1936), such zygotes were not only found intracellularly but also, possibly artefactitiously, freely within the intestinal lumen among the residual debris of degenerate blood. At the end of hibernation, in June, slightly before and during the moulting of the nymphs to adult H. detritum, the encysted zygotes transformed, emerged from their cysts and were found within the intestine. Although Sergent et al. (1936) actually demonstrated ookinetes they were not able to distinguish them from zygotes and reported "On n'a pas observé des formes pouvant être rapportées avec sûreté a des ookinètes". Three to four days after the nymphs moulted and attached on a fresh bovine, parasites were found within salivary gland acinar cells as young sporonts which grew and broke up into sporoblasts within which multitudes of sporozoites appeared. Sergent, Donatien, Parrot and Lestoquard (1936a) reported that moulted infective

H. detritum ticks could transmit Mediterranean Coast fever to a susceptible animal 60 hours after feeding on it as a fresh host.

Schein (1975) studied the development of Theileria annulata in the midgut and haemolymph of Hyalomma anatolicum excavatum. He observed that at the time of repletion, a variety of developmental stages were detectable within the lumen of the gut. Apart from singular intraerythrocytic piroplasms, many free round forms, 3 μm in diameter, with marginal nuclei, were present. Some of the round forms developed spiky structures approximately 3-4 μm long at their periphery. After four to six hours, the spiky forms elongated into a slender, spine-like shape, having single, oval, centrally-located nuclei, an apical spiky structure and a tail-like posterior end. Some of these forms became multinucleate, with up to four nuclei, and developed into microgametes about 12 μm long. Other round forms, 3-4 μm long and regarded as macrogametes, had centrally-located, elongate nuclei and a cloudy, dispersed cytoplasm. Schein (1975), like Sergent et al. (1936) did not observe syngamy. He, however, reported that, three to five days following repletion, the macrogametes increased steadily in size and developed into ovoid zygotes. On the 13th day after the ticks fed on infected blood, the zygotes began to invaginate from the periphery inward almost to the middle and transformed into ookinetes which measured 18 μm long and 5 μm at the broadest part. On the 17th day post repletion they penetrated the gut wall and were detected within the haemolymph. Schein (1975) observed that, from the 18th day after engorgement, the ookinetes invaded salivary gland acinar types II and III and started rounding up into sporonts.

Mehlhorn, Weber, Schein and Buscher (1975) undertook an ultrastructural study of the developmental stages of T. annulata in the intestine and haemolymph of H. anatolicum excavatum nymphs. Like Sergent et al. (1936) and Schein (1975), they reported the presence of sexual stages in the gut lumen of ticks immediately after repletion. From the 1st-6th days after the nymphs dropped engorged, numerous microgamonts and microgametes were observed in the lumen. The microgamonts were spindle-shaped, measured 8-12 μm long and 0.8 μm in diameter in their middle region. They had a stiletto-like apex, several flagellate protrusions, 4-5 μm long, and a slender posterior pole. The microgametes were cord-shaped, 12 μm long and contained a central nucleus and several microtubules running from one end to the other. Mehlhorn et al. (1975) believed, but did not demonstrate, that gamogony occurred in the intestine of H. anatolicum excavatum nymphs. Ookinetes, 20 μm long and 4-5 μm in diameter, were found in the haemolymph of ticks 14-19 days after repletion. They neither discussed the zygote stage nor did they describe stages subsequent to the ookinete development.

Mehlhorn and Schein (1977) studied the development of kinetes of T. annulata in H. anatolicum excavatum by electron microscopy. They reported the presence of zygotes within the intestinal cells of nymphs five days after they engorged on infected blood. Like Sergent et al. (1936), Schein (1975) and Mehlhorn et al. (1975), they failed to observe fertilization. The zygotes grew in size and reached a diameter of 10 μm on the 12th day post repletion. They had a spherical, non-chromocentric, eccentrically-located nucleus of 4-5 μm in diamter. The cytoplasm contained numerous vacuoles.

The development of kinetes within the zygotes commenced on the 10th day post repletion and was depicted by the protrusion of a portion of the zygote cytoplasm and nucleus into a large adnuclear vacuole. The vacuolar membrane was reinforced by two newly formed membranes. As development proceeded, the large vacuole extended and the protrusion became considerably enlarged by the intake of more cytoplasmic and nuclear material of the zygote so that finally, only a small zone of the cytoplasm remained around the intravacuolar differentiating kinete. Around the 12th day post repletion, the remaining narrow cytoplasmic bridge ruptured thus freeing a club-shaped kinete which started moving and emigrated from the intestinal epithelial cells. The parasite was surrounded by a pellicular complex comprising an outer and two inner membranes, and had numerous micronemes, mitochondria, subpellicular microtubules, a polar ring and a number of microtubules posterior to the polar ring. From the 17th day post repletion, a large number of kinetes were found moving in the haemolymph. By the 18th day after engorgement, kinetes left the haemolymph and penetrated salivary gland cells of acinar types II and III.

Schein and Friedhoff (1978) not only studied the developmental stages of T. annulata in the haemolymph but also in the salivary glands of H. anatolicum excavatum nymphs. They demonstrated numerous fully differentiated kinetes, 17.6 µm long, in the haemolymph of the nymphs 17-20 days following engorgement. From the 18th day after engorgement, just before the nymphs moulted to adult H. anatolicum excavatum, kinetes were found in salivary gland cells predominantly in type III acini and less frequently in type II acini. Once intracellular, the kinetes transformed into round multinucleate

sporonts of approximately 10 μm in diameter. The sporonts grew to a size of 20 μm after several nuclear divisions. When the nymphs moulted, the development of the parasite was temporarily arrested until shortly before the adult ticks attached and started feeding on a fresh host when there was not only the activation of the salivary glands but also a renewed multiplicative development of T. annulata stages within acinar cells. The sporonts underwent an intensive multiplication characterised by multiple nuclear divisions and achieved an enormous increase in size. The development of the parasite in feeding adult ticks occurred in four major steps:
(a) the division of sporonts into numerous primary sporoblasts;
(b) the division of primary sporoblasts into secondary sporoblasts;
(c) production of sporozoites by tertiary bodies or cytomeres; and
(d) the degeneration of the cytomeres and their host cells with accompanying release of sporozoites. The development of the parasite within the salivary glands was asynchronous so that in different host cells, the parasites were found at markedly variable stages of development. Schein and Friedhoff (1978) reported that they did not determine the susceptible acinar cell types, possibly e- and d-cells.

2.3.2 In the bovine host

2.3.2(a) Theileria parva: The type of cell susceptible to T. parva infection has been a subject of controversy for a long time. According to Wilde (1967), two main possibilities existed: that the sporozoites, on injection, enter a cell type of the skin or subcutaneous tissue, possibly histiocytes, develop there for some time and subsequently

migrate to lymphoid cells; or that they are carried to the local drainage lymph node and there enter either cells of the lymphatic system or possibly of the reticulo-endothelial system. Daily histological examination of the skin at the site of tick bite (Cowdry and Danks, 1933) had, however, previously failed to show any structures suggestive of T. parva but demonstrated that the medium-sized lymphocytes, first in the lymph nodes and later in all parts of the body are the chief cells infected. Kimeto (1978) undertook an ultrastructural study of cutaneous lesions in calves 120 hours after the attachment of infective Rhipicephalus appendiculatus ticks on cattle and claimed the presence of a stage of T. parva, within a neutrophil, which he called a trophozoite. The structure described by Kimeto (1978) as a trophozoite, however, turned out to be an amorphous mass of phagocytosed material undergoing lysosomal digestion. In a later communication, Kimeto (1980) reported that sporozoites of T. parva, at the site of tick bite, had no predilection for any particular host cell so that they were not only phagocytosed by host leucocytes but also actively invaded erythrocytes as early as five days after tick attachment. He further claimed that there was an extracellular life cycle of T. parva within the bovine tissue. A study of the structures described by Kimeto (1980) as infective particles reveals that they are artefacts and represent, in the majority of cases, dislodged or dislodging neutrophil nuclei or granules, or degenerating platelets.

Brown, Cunningham, Joyner, Purnell, Branagan, Corry and Bailey (1978) reported that the susceptible cells were associated with the

leucocyte fraction of the peripheral blood and not the erythrocytes. Moulton, Krauss and Malmquist (1971) suggested that sporozoites of T. parva were phagocytosed by reticulum cells which then transformed into lymphoblasts. This hypothesis was investigated by De Martini (1972) in an electron microscopic study of the local drainage lymph node early in the course of East Coast fever. He believed that T. parva sporozoites either actively invaded small lymphocytes which consequently underwent blastoid transformation, or lymphocytes undergoing antigenic stimulation in the lymph node. He failed to observe early parasitic forms in phagocytic cells but demonstrated numerous inclusions compatible with initial bodies of Theileria schizonts in lymphoid cells. Since the cytoplasm of the parasitised lymphoblasts neither contained phagosomes nor fibrils of phagosomes usually considered characteristic of reticulum cells, De Martini (1972) believed they were undifferentiated cells of lymphoid series. He, however, was unable to make a clear-cut decision and concluded that the question of the initial cell type invaded by or phagocytosing T. parva sporozoites must remain open pending either an in vitro demonstration of cell invasion by the parasite or improved techniques for the demonstration of early preschizontal stage of Theileria in vivo.

Brown, Stagg, Purnell, Kanhai and Payne (1973) and Brown (1979) described successful infection and transformation of peripheral blood lymphocytes using suspensions of T. parva sporozoites harvested from pre-fed infected R. appendiculatus ticks. Roelants, Buscher, London, Mayor-Withey, Rovis and Williams (1978) suggested that cells transformed by T. parva derived from lymphocytes of the T-lineage.

Duffus, Wagner and Preston (1978) observed that T. parva-infected cells did not possess Ig. Pinder, Withey and Roelants (1981) reported that only a discrete subpopulation, about 7%, of T-lymphocytes was susceptible to transformation by T. parva sporozoites. Pinder and Roelants (1981) observed that this discrete subpopulation was not cytotoxic but could respond to T-cell mitogens and products of the main histocompability complex. Black, Jack, Labor and Newson (1981) observed that infected cells did not resemble resting T-cells because they had a raised expression of P10 and P13 target antigens and also expressed BLA-A antigens. They believed that the infected cell type was not a mature T-cell but that it acquired T-cell differentiation antigens due to gene de-repression following infection. The cyclical development of T. parva in the bovine host is initiated when viable sporozoites are inoculated into a susceptible animal with the saliva of an infected Rhipicephalus appendiculatus during feeding. The first intracellular form of the parasite occurs in the lymphocytes within the local drainage lymph node several days after sporozoite inoculation. The length of the prepatent period is dependent on the volume of an infective inoculum and, thus, the density of T. parva sporozoites inoculated (Radley, Brown, Burridge, Cunningham, Peirce and Purnell, 1974). The shortest time reported in vivo was four days (Jura and Losos, 1980). Schizont-containing lymphoblasts undergo intense mitotic activity (Barnett, 1960), rapidly proliferate and are disseminated systemically so that they are found not only in the various lymphoid organs but also in non-lymphoid body tissues. That the macroschizont divides into two during host cell division so that each daughter cell contains a

daughter schizont was first postulated by Reichenow (1941) and later confirmed *in vitro* by Hulliger, Wilde, Brown and Turner (1964). Hulliger *et al.* (1964) and Hulliger (1965) observed that the individual schizont nuclei divided by binary fission, that the division was not simultaneous and occurred in the interphase cell as well as during all stages of mitosis. Schizont nuclear division was demonstrated by the ultrastructural studies of Jarrett and Brocklesby (1966). The growth rate of *T. parva* macroschizonts in cattle was studied by Jarrett, Crighton and Pirie (1969) and expressed by the term T_{10} i.e. the length of time taken for an observed tenfold multiplication of macroschizonts in the lymph node contralateral to the local drainage lymph node. They induced infections of varying severity by applying 10, 100 and 1000 *T. parva*-infected *Rhipicephalus appendiculatus* ticks to the ears of susceptible cattle. Counts of macroschizonts were made so that daily increases of the parasite could be determined. In the group of cattle infested with ten ticks, small numbers of macroschizonts were detected on Day 16 post infection and the geometric means of the macroschizont indices on Days 17-20 were 2.9, 16.4, 27.3 and 30.3. In the group infested with 100 ticks, geometric means of the macroschizont indices on Day 14-17 were 5.3, 13.5, 33.6 and 52.4. In the third group where 1000 ticks were used to infest susceptible cattle, the macroschizont index values for Days 11-14 were 2.5, 4.5, 12.7 and 21.0. Jarrett *et al.* (1969) observed that in the three experiments, there was a linear relationship between the logarithm of the macroschizont index and time, and the growth rate of the parasite could be calculated from the slopes of the regression lines. They reported that there was no significant

difference in the growth rate of the parasite in the three dosage levels and claimed that the growth rate of T. parva macroschizonts was independent of the size of the dose of infective particles. From their results, Jarrett et al. (1969) erroneously concluded that T. parva macroschizonts increased tenfold every three days irrespective of the concentration of sporozoites inoculated into susceptible cattle. Their results could be attributed to the variable infection rates in the individual ticks. Radley et al. (1974) undertook a similar study but used material containing a uniform suspension of T. parva sporozoites obtained as described by Purnell, Brown, Cunningham, Burridge, Kirimi and Ledger (1973). Their findings were at variance with those of Jarrett et al. (1969). They reported that the rate at which macroschizonts increase in East Coast fever susceptible cattle is related to the volume of the stabilate and therefore to the number of infective particles received by an animal so that the growth rate of any strain of Theileria varies with the size of the infective dose. For example, for Theileria parva (Muguga) at dilutions 10^{-2}, 10^{-1}, 10^{0}, 10^{1} and 10^{2}, the number of days required for a tenfold growth were 2.76, 2.81, 2.61, 2.57 and 2.17 respectively. Radley et al. (1974) also observed that the rate of multiplication of T. parva was not only dose-dependent, but was also subject to modification by a combination of factors dependent largely on the reaction of the recipient animal. These factors, they observed, either eventually destroyed the parasites or failed to contain them as a result of which the animal died. This multiplicative phase of the macroschizont is followed, often several days following the onset of temperature reaction in a case of East Coast fever, by another

stage of the parasite, the swarming form (Meyer, 1910) or the microschizont, whose presence was soon followed by the appearance of intraerythrocytic piroplasms. Ultrastructural studies of Jarrett and Brocklesby (1966) demonstrated that a T. parva microschizont represented a parent schizont undergoing schizogonous division surrounded by offspring merozoites in different stages of budding off. These authors, however, wrongly believed that microschizonts were produced inside phagocytic cells after the latter phagocytosed macroschizonts. Buttner (1967) and Schein, Mehlhorn and Warnecke (1978) described the schizogonous process by which merozoites arise from T. parva macroschizonts. Schein et al. (1978) gave a detailed account of the process. The nuclei of the schizont became arranged at the periphery of its pellicle so that the parasite appeared rosette-like. During the last schizont nuclear division, a merozoite-anlage appeared above each nuclear pole. At the apical pole of such an anlage, an inner pellicular complex forming a polar ring system to which subpellicular microtubules were anchored became visible. Three or four rhoptries extended into the opening of the polar ring. A few micronemes were observed scattered at the apical pole of each anlage. Finally the merozoites became free almost simultaneously from the residual body representing the parent schizont. Beginning from the merozoite-formation phase, the parasite appeared as "microschizonts" in light microscopy. Liberated merozoites invade host erythrocytes and develop into intraerythrocytic piroplasms. The appearance of piroplasms is time-dependent and not influenced by the dose of the inoculum (Jarrett et al., 1969). T. parva piroplasms usually appear, irrespective of the dose of

infective particles, around Day 12 (Jura and Losos, 1980) after inoculating a susceptible animal. A fresh cyclical development of T. parva may be initiated if larvae or nymphs of Rhipicephalus appendiculatus feed on cattle blood containing piroplasms.

2.3.2(b) Theileria annulata: The developmental cycle of T. annulata in bovine tissues, although generally similar to that of T. parva, has received very little attention and thus lacks the details encountered in the case of the latter parasite. When the ears of susceptible cattle are infested with Hyalomma ticks infected with T. annulata, macroschizonts may be detected in smears of needle biopsy material obtained from the parotid lymph node 7-18 days (Sergent, Donatien, Parrot and Lestoquard, 1945) or 7-12 days later (Gill, Bhattacharyulu and Kaur, 1977). Sergent et al. (1936; 1936a; 1936b; 1945) reported that T. annulata schizonts were originally found in lymphoid cells but were later disseminated systemically and detected within phagocytic reticulo-endothelial cells and fibrocytes of the adjoining connective tissue. Brown (1979) demonstrated the infection and transformation of lymphoid cells in vitro by T. annulata sporozoites. Brown and Gray (1981) demonstrated successful infection and transformation of fibroblastic cells with T. annulata sporozoites. The infected fibroblasts could be maintained indefinitely in vitro. Gill et al. (1977) observed that the severity of Mediterranean Coast fever depended not only upon the virulence and pathogenicity of the strain of the parasite but also on the volume of the inoculum and hence the density of Theileria annulata sporozoites. Macroschizont-containing lymphoid cells increased in number and, about 1-3 days later, were disseminated

systemically thus initiating a temperature reaction (Sergent et al., 1945). Gill et al. (1977), however, reported that systemic involvement may be observed between the 5th and 12th days after infective Hyalomma ticks feed on a susceptible animal, a point which supported the quantum of infection hypothesis (Wilde, 1967). Sergent et al. (1945) observed that the schizonts gave rise to merozoites which penetrated erythrocytes 3-4 days after initial temperature reaction. It has been observed in this laboratory that the appearance of T. annulata piroplasms is time-dependent and invariably occurs around Day 9 following the inoculation of cattle with sporozoites. Du Toit (1918) observed that T. annulata piroplasms multiplied within host erythrocytes. Division into two produced two daughter piroplasms (Du Toit, 1918), or alternatively the parasites divided into four (Sergent et al., 1945) resulting in cross forms, in which four minute pear-shaped individuals radiated from a central point. The latter authors observed that in recovered animals the piroplasms may persist up to 11 years.

2.4 Comparable and contrasting aspects of T. parva and T. annulata infections

2.4.1 Comparable aspects

Both Theileria annulata (Sergent et al., 1936; Mehlhorn et al., 1975; Schein, 1975; Schein et al., 1975) and T. parva (Cowdry and Ham, 1932; Mehlhorn and Schein, 1976; Schein et al., 1977; Schein, Mehlhorn and Warnecke, 1978) develop sexual stages within the gut of vector ticks on the 1st-6th days after the immature ticks feed on blood containing piroplasms. No fertilization has, however, been

confirmed in the development of both parasites. In both T. annulata (Schein, 1975) and T. parva (Cowdry and Ham, 1932; Schein et al., 1977), following a presumed fertilization, zygotes appear within epithelial cells of tick gut about six days after repletion. In T. annulata (Schein and Friedhoff, 1978) as well as in T. parva (Cowdry and Ham, 1932) sporonts are detectable within the salivary gland cells before ticks moult, about 18 days and 23 days post repletion respectively. The development of the sporonts to mature infective sporozoites takes place in both parasites after infected, moulted vector ticks have attached and fed on a fresh host.

Both T. parva (Gonder, 1911b) and T. annulata (Brown, 1979) develop as schizonts in lymphoid cells. The rate of multiplication of the schizont stage of T. parva (Radley et al., 1974) as well as T. annulata (Gill et al., 1977) is dependent on the quantum of infection. In both parasites, the development of the intraerythrocytic piroplasms is time-dependent and fever is a characteristic symptom in East Coast fever as well as in Mediterranean Coast fever. Leukopaenia is a feature of both diseases.

2.4.2 Contrasting aspects

Microgamonts of T. parva (8–10 μm) are somewhat shorter than those in T. annulata which are 8–12 μm long (Schein et al., 1977). The spine of the microgamonts of T. parva (2 μm) is only half as long as in T. annulata (4 μm). The transformation of zygotes of T. annulata to kinetes starts only several days after the supposed fertilization, i.e. on the 9th–10th day following repletion whereas in T. parva the change takes place much later, about the time of

moulting, i.e. from 15-20 days after engorgement (Cowdry and Ham, 1932; Mehlhorn et al., 1978). In T. parva sporogony, depicted by nuclear division, starts in the kinetes during their migration to the salivary glands as opposed to the case of T. annulata in which sporogony begins within acinar cells (Mehlhorn et al., 1978). Whereas in T. annulata maximum production and maturation of infective sporozoites takes places 2-3 days after moulted ticks start feeding on a fresh host (Schein and Friedhoff, 1978), in T. parva 4-5 days feeding is optimal (Cowdry and Ham, 1932). Piroplasms of T. parva invariably appear around 12 days post infection (Jura and Losos, 1980) while those of T. annulata have been observed in this laboratory to consistently develop around Day 9 after inoculating a susceptible animal. Recovery from East Coast fever confers a solid, sterile immunity on an animal (Du Toit, 1928) whereas in Mediterranean Coast fever, intraerythrocytic piroplasms persist in recovered cases (Sergent et al., 1936) so that splenectomy of such cattle results in a recrudescence of T. annulata stages even as late as 11 years following recovery. While in East Coast fever, over 80% of piroplasms are rod-shaped and less than 20% round or oval, in Mediterranean Coast fever, only about 20-30% are rod-shaped and 70-80% are round or oval (Henning, 1956). Severe anaemia accompanied by polychromatophilia, basophilic stippling, haemoglobinuria and mild icterus usually develops in Mediterranean Coast fever (Sergent, Donatien, Parrot, Lestoquard, Plantureux and Rougebief, 1924; Sergent et al., 1945; Hooshmand-Rad, 1976) while in T. parva infection anaemia, if present, is invariably mild, normocytic, normochronic and non-responsive (Brown, Wilde, Hulliger, 1965; Wilde, 1966; Maxie,

Dolan, Jura, Tabel and Flowers, 1982). Disseminated intravascular coagulation, as indicated by positive protamine paracoagulation tests, prolonged prothrombin and partial thromboplastic times, and thrombocytopenia, has been demonstrated in T. parva infection (Maxie et al., 1982) as an important intermediary leading to death. T. annulata piroplasms divide within erythrocytes (Du Toit, 1918; Sergent et al., 1945), but those of T. parva do not (Wenyon, 1926; Reichenow, 1940). Not only do the T. annulata and T. parva piroplasm isoenzyme patterns differ (Melrose and Brown, 1979) but also the electrophoretic mobilities of their schizont glucose phosphate isomerase (Melrose, Brown and Sharma, 1980; Musisi, Kilgour, Brown and Morzaria, 1981). The same enzyme differentiated T. parva from T. annulata when lysates of their sporogenous stages were examined for enzyme polymorphism by thin layer starch gel electrophoresis (Melrose, Walker and Brown, 1981). Whereas T. parva infection is characterised by a massive interstitial pneumonia accompanied by an accumulation of yellow, fibrin-containing fluid exudate in the upper respiratory tract, lung parenchyma and pleural cavity (Losos and Jura, in preparation), the only lung involvement invariably observed in T. annulata infection is a very mild edema usually with no appreciable change in the consistency of the organ (Gill et al., 1977).

2.5 In vitro Theileria-cell cultivation

2.5.1 Development of Theileria-cell culture

2.5.1(a) Theileria annulata: The development of in vitro Theileria-cell cultivation, initiated by Tsur-Tchernomoretz (1945), has not only been reviewed by Pipano (1977) but also updated by Brown (1979a, 1980). Tsur-Tchernomoretz (1945), using a method modified by Jacoby

(1944), claimed the first success when he grew and achieved multiplication, *in vitro*, of the intralymphocytic, schizont stage of the Tova strain of *Theileria annulata*. Small fragments of infected calf spleen or lymph node were placed on sterile coverslips and covered by a drop, each, of calf plasma and chicken embryonic extract. After the drops coagulated, the coverslips were sealed to the bottom of a large carrel flask by a drop of plasma and embryonic extract. Three to five ml of a mixture of 30-40% calf serum in Tyrode solution was then added and the flask closed and incubated. The culture survived for 12 days but did not multiply in this medium. Addition of fragments of fresh spleen to the culture led to the prolonged survival up to 15-18 days but did not induce multiplication. Tsur-Tchernomoretz (1945) later supplemented the above medium with 3 µg/ml glutamine, 0.6 µg/ml pyridoxine, 4 µg/ml inositol and 0.04 µg/ml riboflavin and achieved the multiplication of *T. annulata*-lymphoid cultures which survived for a period of two months. Tsur-Tchernomoretz (1947) undertook more experiments but cultures were viable for only one to three weeks. Further multiplication and survival of the cells were impeded by necrosis so that attempts to obtain serial passages failed during the initial subcultures. Tsur-Tchernomoretz (1953) set up another lot of experiments using the same method and medium as before and once more achieved growth and multiplication of schizont-lymphoblast cultures for two months in ten successive fragments of normal calf spleen. He not only observed that schizonts remained infective for calves after four passages, but also that they grew in juxtaposition to spleen fragments from recovered animals or in medium enriched with 40% serum obtained from such animals.

Brocklesby and Hawking (1958) reported successful cultivation of Theileria annulata using a technique previously described by Hawking (1945; 1951). They attached implants, 1-2 cu.mm, obtained from infected spleen, lymph node or other tissue, to coverslips by a drop of plasma. The coverslips had already been attached to the floor of either a carrel flask, flat medicine bottle or petri dish. They used two media, a basic and an enriched. The basic medium consisted of 40% ox serum, 5-10% chick embryo extract, and 50-55% Hanks' salt solution with 0.002% phenol red and supplemented with growth factors. The enriched medium was a synthetic mixture No. 199 described by Morgan, Morton and Parker (1950). After addition of fluid medium to the petri dishes with implants, they were placed in a sealed box containing 5% carbon dioxide in 95% air at 37°C. The carrel flasks and flat medicine bottles were also incubated at 37°C. Brocklesby and Hawking (1958) observed that T. annulata-lymphoid cultures grew and multiplied for over 59 days and that the cultures were infective for cattle when tested after 17 and 42 days. The method and medium used by Brocklesby and Hawking (1958) resemble very closely what is currently in use and should have supported relentless growth of the schizont-lymphoblast cultures. That this did not happen is surprising and a possible explanation is expressed by Pipano (1977) who thought that the coagulated plasma covering the implants could have blocked the proliferation and spread of the schizont-infected cells over the available surface of the flasks. Tsur-Tchernomoretz and Pipano (1959) cultivated T. annulata schizont-infected spleen tissue fragments on coverslips in a clot of bovine plasma and chick embryo extract by the addition of rodent spleen

tissue implants. The parasite underwent growth and multiplication through six passages in the course of two months. Tsur and Adler (1963) set up experiments using infected bovine liver, spleen, lymph nodes and coagulated white blood which were cut up with scissors, digested in 0.25% trypsin solution and centrifuged. The pellet of cells containing T. annulata schizonts were resuspended at 5×10^5 cells/ml in Earle's solution containing bovine serum, antibiotics and yeast. Five to ten ml of this suspension were transferred into flasks which had coverslips fixed to their floors, and which were subsequently stoppered and incubated at 38°C. The supernate was discarded and replaced every three to four days by fresh Earle's solution. Tsur and Adler (1963) observed that (a) three series of cultures from infected bovine tissues continuously showed lymphatic cells and schizonts on the coverslips and supernatant medium, (b) subcultures of cell pellets obtained by centrifuging supernatant medium of two-month old tissue cultures showed good growth and multiplication without the addition of normal cells, (c) no fibroblasts were infected, and (d) three months after their establishment, the cultures were still continuing to grow. Tsur, Adler, Pipano and Senft (1964) used a similar technique and maintained T. annulata in culture for two years. Hulliger, Wilde, Brown and Turner (1964) made observations on Theileria-infected cells growing in monolayer. The cells had been isolated from bovine spleen, lymph nodes or blood obtained from animals infected with the Tova strain of Theileria annulata and were grown in association with a cell line of baby hamster kidney (BHK), isolated by Macpherson and Stoker (1962). Hulliger et al. (1964) reported a successful mass

cultivation of T. annulata over a period of four months, the time during which no signs of cytopathogenic activity by the parasite was detected. They put forward a concept that the distribution of Theileria into both daughter cells during mitotic division of the host cell was responsible for the increase in numbers of infected cells. Hulliger (1965) undertook further investigation using lymph nodes, spleens and buffy coat from animals infected with Theileria annulata. Her growth medium was composed (by volume) of 10% calf serum, 10% tryptose phosphate broth and 80% Eagle's minimum essential medium modified according to Stoker and Macpherson (1961). Streptomycin, penicillin and neomycin were added in a final concentration of 100 µg/ml, and mycostatin at 25-50 units/ml. Cells from spleen and lymph nodes were collected by suspending pieces of tissue in a petri dish containing complete growth medium as above, cutting them up with scissors so that finally, a suspension of lymphoid cells was obtained. The concentration was adjusted to 10^6 cells/ml. Buffy coat cells were separated from venous blood containing heparin at 50 units/ml. Blood was centrifuged first at 2,500 rpm for eight minutes and then at 2,000 rpm for five minutes. The buffy coat layer was resuspended in complete growth medium at 10^6 cells/ml. Cell suspensions from tissues and buffy coat were each dispensed in 4-oz medical flasks at 10^6 cells/ml and mixed with freshly trypsinised suspension of BHK cells at 10^5 cells/ml and incubated at 37-40°C. Medium changes and subculturing were carried out once per week in the first week of cultivation but changed to twice per week. Hulliger (1965) observed that in different experiments, cultures were propagated continuously for 205, 105 and 111 days. She reported

that no continuous multiplication was obtained if only lymphoid cells were cultivated without a supporting feeder layer such as BHK or fibrocytic cell type. She, however, believed that BHK was superior to fibrocytic cells because (a) they manifested rapid growth which provided favourable metabolic conditions during the early stages of cultivation of lymphoid cells, (b) they adopted a whirl-like fibrocytic pattern which helped the bovine lymphoid cells to attach to the glass, where multiplication was favoured. She consequently concluded that Tsur and Adler (1963) were able to cultivate T. annulata from trypsinised spleen probably because they obtained two types of cells, a fibrocytic bovine cell type which had a function similar to BHK and the lymphoid, Theileria-infected cells which multiplied continuously. Tsur and Adler (1965) grew T. annulata from cells obtained from the peripheral blood of cattle in the overt stage of Mediterranean Coast fever using the monolayer technique. The blood was transferred into a sterile tube containing heparin, lightly centrifuged and the supernatant plasma discarded. Chicken embryo extract was then layered onto the surface of buffy coat and incubated at $37^{\circ}C$ for three hours to hasten coagulation. The buffy coat was then separated, washed in Earle's solution, stirred in 0.25% trypsin solution for a few hours then mixed with bovine serum and centrifuged. The pellet was washed and resuspended in 10 ml Earle's solution containing bovine serum and antibiotics, pH 7.2, at $2\text{-}3 \times 10^{6}$ cells /ml and cultivated in flasks which had coverslips attached to the floor with plasma and embryonic extract. Growth and multiplication occurred and cultures were maintained for more than three months. Tsur and Pipano (1966) maintained T. annulata-

lymphoid cultures in association with monolayers for nine months by changing medium twice weekly and 1-2 monthly subculturing. They reported that a strain of T. annulata maintained continuously in culture in vitro for 20 months completely lost its virulence so that when it was inoculated into susceptible cattle, the animals became immune without showing any signs of disease such as schizonts, piroplasms or thermal reaction. Pipano and Tsur (1966) established cultures from T. annulata-infected liver, spleen and lymph nodes as well as white blood cells using methods described earlier (Tsur and Adler, 1963; 1965). Supernatant medium from the cultures, containing 10^6-1.5 x 10^6 schizont-infected cells, was administered subcutaneously to susceptible cattle. In seven out of eight calves inoculated with schizont-infected cells grown in tissue culture for 3, $4\frac{1}{2}$ and $6\frac{1}{2}$ months, there was a severe clinical disease accompanied by a marked rise in schizont and piroplasm levels and three of the calves died. Nine calves inoculated with infected cells maintained in culture for seven months manifested clinical disease but no mortality. Eight out of 17 cattle inoculated with infected cells maintained for $9\frac{1}{2}$-12 months in tissue culture showed mild reaction with few parasites while the rest of the animals neither showed any clinical signs nor could schizonts be found in liver smears. Sixteen cattle inoculated with T. annulata-infected cells maintained in tissue culture for 21-35 months neither showed clinical signs nor parasites. All the vaccinated cattle along with seven susceptible calves were challenged 50 days post inoculation. Whereas vaccinated animals were immune to challenge, two out of seven susceptible controls died and the remainder showed mild to severe temperature reactions accompanied

by occasional parasites. Pipano and Tsur (1966) therefore demonstrated that T. annulata maintained in vitro was infective for cattle, but lost its virulence correspondingly with increasing days in culture. When this avirulent schizont stage of the parasite was introduced into cattle, they became immune to challenge with infected blood obtained from animals suffering from acute Mediterranean Coast fever. Zablotskyi (1966; 1967) cultivated T. annulata using material obtained from infected spleen and lymph nodes. He reported that attenuation in the virulence of T. annulata schizonts occurred as early as 8-23 days of maintenance in tissue culture. Hooshmand-Rad and Hashemi-Fesharki (1968) for the first time grew T. annulata-infected cells in suspension culture without a fibroblastic feeder layer. Pipano and Israel (1971) carried out trials with wild field strains of T. annulata and obtained results which corroborated the observations of Pipano and Tsur (1966) that complete attenuation was accompanied by loss of capacity to produce erythrocytic forms. Brown (1979) reported infecting and transforming peripheral blood lymphoid cells with T. annulata sporozoites obtained from pre-fed ticks which were surface-sterilised and ground up in medium containing bovine plasma albumin, antibiotics and mycostatin. He reported that the resultant cultures exhibited the first intracellular forms of T. annulata within 24 hours, macroschizonts on the second day and could be subcultured as early as the fourth day after infection. The cultures could be maintained for over three years. Brown and Gray (1981) successfully infected bovine fibroblastic cell lines using T. annulata sporozoites and observed that the infected cell lines could be maintained indefinitely in vitro.

2.5.1(b) Theileria parva: The first attempt to cultivate T. parva in vitro was made by Tsur-Tchernomoretz, Neitz and Pols (1957) who used the plasma clot technique previously employed by Tsur-Tchernomoretz (1945) to grow T. annulata-infected lymphoblasts. Although Tsur-Tchernomoretz et al. (1957) maintained T. parva-lymphoid cultures for 15 days, active growth and multiplication were only observed during the first ten days. Cell proliferation persisted for 20 days in cultures to which streptomycin, penicillin, aureomycin and plasmoquin had been added. Brocklesby and Hawking (1958) maintained T. parva-infected lymphoid cells for 14 days in tissue culture. Only limited multiplication was observed during the first few days, and no infected cells were found surviving after 14 days. The authors observed that T. parva schizonts grew principally in lymphoid cells. Hulliger et al. (1964) and Hulliger (1965) used the BHK cell line as a feeder layer and reported a successful mass cultivation of T. parva in lymphoid cells isolated from spleen, lymph node or blood from infected cattle. It was, however, later shown by in vivo and cross-immunity studies (Wilde, 1967) that Hulliger et al. (1964) and Hulliger (1965) only succeeded in cultivating T. annulata but not T. parva. Susceptible cattle inoculated with a suspension of T. parva-infected cells derived from tissue culture invariably manifested a clinical episode varying from establishment of the parasite in the lymph nodes with a mild temperature reaction to a fatal syndrome. When recovered cattle were challenged with tick-transmitted T. parva, they proved fully susceptible. If, on the other hand, recovered animals were challenged with T. parva-infected tissue culture material, they were found to

be immune and withstood the challenge, thus indicating that tissue culture material was immunogenic. In another experiment, cattle recovered from an infection with T. parva-infected, tissue culture cells provided material for immunological tests in which blood-transmitted T. annulata, tissue culture-derived T. annulata, T. parva from tissue culture and tick-transmitted T. parva as well as natural and tissue cultured T. lawrencei were used. Cross-immunity existed between T. parva from tissue culture and both natural and tissue culture-derived T. annulata as well as natural and tissue cultured T. lawrencei. No cross-immunity was observed between naturally transmitted and tissue culture-derived T. parva materials. The first breakthrough in the cultivation of T. parva was achieved by Malmquist, Nyindo and Brown (1970) who used portions of spleen collected from calves killed at the peak of East Coast fever as the source of infected cells. Cultures were established in Eagle's minimum essential medium (MEM) with Earle's salt base, containing 0.1 g L-β-asparagine/litre, 100 units/ml penicillin, 100 μg/ml dihydro-streptomycin and 20% foetal calf serum. No feeder layer was used and subcultures of transformed cells growing in suspension were made at three- to four-day intervals. The cells were sedimented by low speed centrifugation, resuspended in fresh growth medium to achieve a 1:3 to 1:10 dilution, and reintroduced into culture vessels. Malmquist et al. (1970) observed that spleen cells from cattle experimentally infected with T. parva could be established as cell lines in which nearly 100% of the cells were parasitised. They reported that feeder layers may be essential for the establishment of infected cell lines in vitro but were not necessary for their maintenance.

The authors not only maintained T. parva-infected cell lines for up to ten months but also noted that there were no factors limiting the life span of the infected cells in vitro. The transformation, they observed, was initiated by the parasite which also stimulated the cells to undergo an indeterminate number of replicative cycles so that a true symbiotic relationship existed in vitro between the cell and the parasite. Brown et al. (1973) established T. parva cultures using (a) normal bovine lymphoid cells obtained from heparinised blood according to Aalund, Hoerlein and Adler (1970) and teased spleens and mesenteric lymph nodes, and (b) in vitro tick feed material (Cunningham, Brown, Burridge, Musoke, Purnell, Radley and Sempebwa, 1974) and ground up tick supernate (Purnell, Brown, Cunningham, Burridge, Kirimi and Ledger, 1973). The cells were established as monolayers in small "T" flasks using 6 ml MEM containing 0.1 g/litre L-β-asparagine, 20% foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin sulphate and 0.29 g/litre L-glutamine. After normal cells were maintained in cultures for 2, 9 and 23 days, the supernatant medium was recovered and the cultures inoculated with either in vitro tick feed or ground up tick supernate. After inoculation, all cultures were overlaid with 6 ml of fresh growth medium containing 25 units/ml nystatin and incubated at 37°C. The medium in all cultures was changed once a week. Infected lymphoid cells were observed 26 days after inoculation in the 9- and 23-day mesenteric lymph node cultures and appeared as small clumps of refractile cells in suspension or associated with lymph node monolayer of the relevant culture. Schizonts were detected 48 days after inoculation in the cultures

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established from heparinised blood. This infection, however, did not last as it could not be maintained. Malmquist and Brown (1974) established lymphoblastoid cell lines infected with T. parva from lymph node biopsy samples from cattle reacting to East Coast fever using homologous feeder layers. Bovine embryonic spleen (BESP) cell lines, used as feeder layers, were established according to Malmquist, Van der Maaten and Boothe (1969). The growth medium and methodology were as described previously (Malmquist et al., 1970). Malmquist and Brown (1974) reported that the use of homologous feeder layers made the establishment of T. parva cultures relatively simple and yielded a high percentage of positive results in contradistinction to the cases where cells were cultured independent of feeder layers. An attempt by Brown and Gray (1981) to infect fibroblastic cell lines with T. parva did not succeed and the infection was lost within three passages.

2.5.2 Use of Theileria cell culture to study host-parasite interactions

The development of methods for continuous culture in vitro of Theileria parasites has made real the possibility of understanding the complex relationship between these protozoa and their host cell. The invasion of cattle cells by T. parva sporozoites in vitro was studied at light microscope level by Stagg, Dolan, Leitch and Young (1981). Bovine peripheral blood leucocytes were separated using a technique based on the methods of Thorsby and Bratlie (1970) and Carlson and Kaneko (1973). Ground up tick supernate (Purnell et al., 1971) separated from 4-day fed Rhipicephalus appendiculatus was concentrated by centrifugation at 1,200 xg for 20 minutes and resuspended

in MEM with 25 mM HEPES buffer plus bovine albumin 0.7% (w/v), MEM/BPA 0.7% at an equivalent of 10-15 ticks/ml. Cultures were set up by mixing 1 ml of sporozoite suspension with 1 ml of cell suspension adjusted to 10^6 cells/ml and allowed to stand at 25°C for 20 minutes. They were then made up to 5 ml with MEM containing 20% foetal calf serum and introduced into 25 cm^2 flasks containing a previously established monolayer of BESP cell line (Malmquist et al., 1969). Samples were taken for Giemsa-stained centrifuge preparations at 1, 8 and 20 hours and thereafter daily. Stagg et al. (1981) also obtained material for living preparations examined under Nomarski Interference contrast microscopy at the same intervals as above. They observed that from one hour after mixing sporozoites with cells, the parasites surrounded and attached to about 25% of the cattle cells which were of medium size, had an extensive cytoplasm and as many as 50 attached sporozoites. Rarely by one hour, but commonly by 20 hours, T. parva sporozoites were detected intracellularly where they became associated with the Golgi apparatus, began to grow and adopted a pyriform shape. Macroschizonts were observed as late as three days after infection. They reported that multiply infected cells did not survive thus implying that sporozoites, rather than toxic tick material, were responsible for cell death. Singly infected cells, which began to divide from Day 3 onwards, Stagg et al. (1981) believed, eventually resulted in established T. parva-infected lymphoblastoid cell lines. Fawcett, Doxsey, Stagg and Young (1982b) studied the events associated with the entry of T. parva sporozoites into bovine lymphocytes in vitro by electron microscopy. Methods used for the separation of

lymphoid cells and sporozoites as well as for the establishment of cultures were described by Stagg et al. (1981). Samples containing 4×10^7 cells were centrifuged at 300 xg for five minutes and the resultant pellets processed for examination by electron microscopy. Fawcett et al. (1982b) reported that T. parva sporozoites made contact with bovine lymphocytes at any point on their surface, that they entered the cells by endocytosis which was completed in less than 10 minutes and did not require energy. They not only observed that the investing host cell membrane acquired during endocytosis had disappeared 24 hours after entry but also that the rhoptries and micronemes were also eventually lost. The disappearance of the host cell membrane was thought to help the parasite evade the host lysosomal system. Fawcett et al. (1982b), however, did not describe the sequential ultrastructural changes the parasite underwent following interiorisation. They did not pinpoint the stage in the development of the sporozoite at which either the investing host cell membrane or the rhoptries and micronemes were lost by the parasite. There was neither a description of how T. parva sporozoites gave rise to the schizont stage, nor were the fine structural changes of the host cell ensuing on infection considered even as late as 72 hours after infection. The observation the authors made that the interiorisation of T. parva sporozoites into cells did not depend on energy was not supported by any data since neither energy metabolic studies nor quantitative comparison of the rates of entry at $2^{\circ}C$ and $37^{\circ}C$ were undertaken.

Hulliger et al. (1964) and Hulliger (1965) used the Tova strain of Theileria annulata to study the mode of multiplication of the

parasite. They observed that in the first few days of cultivation few bovine cells were in mitosis, most of them infected with Theileria schizonts. In the later stages a very high percentage (98-100%) of dividing cells contained parasites so that established cultures consisted of a clone of parasitised bovine lymphoid cells dividing continuously. The parasite was closely associated with the spindle of the dividing host cell and was distributed to both daughter cells during late mitosis. In prophase, schizont nuclei were found in the cytoplasm of the host cell between the host chromosomes; in metaphase, the parasite almost invariably lay between the chromosomes, thus disturbing the symmetry of the equatorial plate; in anaphase, the schizont nuclei were aligned in one or two, rarely more, threads along the fibrils of the spindle, each thread of nuclei being enclosed in a coherent strand of cytoplasm; in telophase, the thread-like schizont was stretched by the dividing cell and in reconstitution there was complete separation of the host cell as well as the schizont so that each daughter cell contained the parasite. The authors also reported that schizont nuclei divided by binary fission and that the division took place not only in the resting host cell but also during all stages of mitosis. Hulliger et al. (1964) and Hulliger (1965) therefore believed that the schizont was merely squeezed into a thread-like structure alongside the host chromosomes and in between the host spindle microtubules, eventually breaking into two as the host cytoplasm constricted and broke off. Although they observed that the schizont nuclei divided by binary fission, the mechanism and nature of this division was not defined. Stagg, Chasey, Young, Morzaria and Dolan

(1980) undertook an electron microscopic study of lymphoblastoid cells transformed by the eland parasite, Theileria taurotragi, or T. parva and erroneously disagreed with Hulliger et al. (1964) and Hulliger (1965) by suggesting that the developing macroschizont became associated with host cell microtubules during interphase and that this association continued during the life of the parasite. While attempting to illustrate synchronisation of the division of Theileria taurotragi and T. parva and that of their host cells Stagg et al. (1980) unfortunately did not show any dividing cells but instead demonstrated a freshly formed, interphase daughter cell in which residual connection still existed between a daughter schizont and spindle microtubules of the host cell. Vickerman (1980) and Vickerman and Irvin (1981) described ultrastructural features of the relationship between the mitotic apparatus of the host cell and the schizont of Theileria parva using in vitro cultured lymphoblasts infected and transformed by T. parva. They observed that during interphase, schizonts were located close to the cell centre (Golgi-centriole region) and were frequently enveloped by host cell endoplasmic reticulum of the annulated lamella type. During centriole replication and outgrowth of the aster, the schizonts became encased in host cell microtubules which lay close to the membrane of the parasite. As the extranuclear spindle elongated, the schizonts became compressed between polar fibres so that with dissolution of host cell nuclear membrane, the schizont was incorporated into the mitotic spindle alongside the chromosomes. During anaphase, the daughter chromosomes were drawn to the poles as the chromosomal fibres shortened, but the schizont merely elongated further along the

continuous polar fibres. The formation of the telophase body and its eventual constriction between daughter host cells concurrently pinched the schizont into two. Like Stagg et al. (1980), Vickerman (1980) and Vickerman and Irvin (1981) believed that host microtubules developed an association with schizonts in the interphase cell. They reported that microtubules merely encased but did not insert on the parasite membrane. Their observation on the synchrony of schizont distribution was in agreement with that of Hulliger et al. (1964). Musisi, Bird, Brown and Smith (1981) not only examined T. parva- but also T. annulata- and T. lawrencei-lymphoblastoid cultures. Their ultrastructural study showed very active Golgi complexes with associated annulate lamellae, prominent cytoplasmic microtubules and an involvement of schizonts with mitotic spindles within dividing lymphoblasts. The annulate lamellae were observed to occur almost invariably in the region of the Golgi complex and closely conformed to the parasite mass. They rarely occurred simultaneously with microtubules. The authors suggested the following hypothetical sequence of events in Theileria-infected lymphoblasts: (a) at prophase, the cell formed annulate lamellae via the Golgi complex, (b) at metaphase, the annulate lamellae became active and contributed towards the formation of cytoplasmic microtubules which joined the parasite and host cell centriole, (c) concurrently the host cell nucleus envelope shared in the formation of microtubules which joined host cell chromosomes and centriole. These microtubules, the authors believed, then lined up to form the mitotic spindle. The parasite was treated as a chromosome and was drawn with the spindle contraction into the respective daughter cells prior

to cytoplasmic division. Irvin, Ocama and Spooner (1982) undertook a study of the cyclical events in bovine lymphoblastoid cells parasitised by T. parva. They reported that macroschizonts incorporated tritiated thymidine when the host cell was in metaphase and not concurrently with the host cell in which incorporation took place during the S-phase. The division of the macroschizonts occurred immediately after they synthesised their DNA, the G2 period being omitted. Irvin et al. (1982) surprisingly concluded that T. parva regulated its own DNA synthesis independent of the host cell. T. annulata maintained in vitro for a long time lost its virulence (Tsur and Pipano, 1966; Pipano and Tsur, 1966) and developed into a successful vaccine modulating the response of the host animal to infection.

CHAPTER THREE

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CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1 Cattle source of peripheral blood lymphocytes (PBL)

Blood for preparing PBL was obtained from Ox No. 46, a five-year old member of a monozygotic pair of twins, kept in a tick-free pen and never exposed to infection with Theileria or other haemoprotozoal parasites at any time.

3.2 Anticoagulant

Acid citrate dextrose (ACD) was used as the anticoagulant and was prepared by mixing 0.8 g citric acid BP, 2.2 g sodium citrate BP and 2.24 g anhydrous dextrose PhEur (all products Analar grade; British Drug House (BDH) Chemicals Ltd., Dorset, Poole, England) in 100 ml deionized distilled water. The resultant solution was sterilised by filtration through a sterile 25 mm polypropylene Swinnex filter holder, type AP25 depth filter and a 0.22 μm MF filter of cellulose acetate and cellulose nitrate material (all products of Millipore Corporation, Bedford, Massachusetts 01730, USA).

3.3 Collection of blood

3.3.1 In ACD

With animal No. 46 restrained in a crush, the area of the skin around the jugular vein was shaved with either a pair of clippers or scissors and thoroughly swabbed with cotton wool wetted in 70% ethanol. Jugular venipuncture was carried out using a sterile 14G x 1½-inch needle and after some blood escaped, 100-ml of blood were aseptically collected into a 100 ml sterile bottle containing 20 ml

of ACD. Mixing of the blood and the anticoagulant was effected during the bleeding by a gentle agitation of the bottle.

3.3.2 Defibrinated blood

The animal was prepared for bleeding as above and venipuncture carried out using a sterile 14G x 1½-inch needle. One hundred and fifty ml jugular blood were collected in a 150-ml conical flask and aseptically defibrinated by continuous stirring with sterile sticks until the smallest possible clot was formed around the sticks.

3.4 Media

3.4.1 RPMI 1640 medium (Moore, Gerner and Franklin, 1967)

The RPMI 1640 medium, buffered with 25 mM N^1-2-Hydroxyethyl-piperazine-N^1-Ethanesulphonic Acid (HEPES) to stabilise and resist rapid pH changes in the medium, was supplemented with 20% foetal calf serum heat inactivated at 56°C for 30 minutes and, just before use, 2 mM/ml L-glutamine (all products of Gibco-Europe, 3 Washington Road, Paisley, PA3 4EP, Scotland). The supplemented medium was treated with 100 iu/ml benzylpenicillin (sodium) B.P. and 100 µg/ml streptomycin sulphate B.P. (both antibiotics from Glaxo Laboratories Ltd., Greenford, England). When supplemented and treated as above, RPMI 1640 was considered a complete growth medium and will be referred to as the complete RPMI 1640.

3.4.2 Minimum essential medium (MEM) (Eagle, 1959)

MEM (Gibco-Europe) was supplemented in various ways depending on the purpose for which it was used.

(i) For the preparation of Theileria sporozoite suspensions

MEM with Hanks' salts and 25 mM HEPES buffer (Cat. No. 041-2370; Gibco-Europe) was supplemented with 3.5% (W/V) bovine plasma albumin (MEM/BPA - Bovine Plasma Albumin - Armour Fraction V - Sigma Chemical Company, Box 14508, St. Louis, Missouri 63178). The solution was sterilised by filtration through a sterile 25 mm polypropylene Swinnex filter holder, with type AP25 depth filter and a 0.22 μm MF filter of cellulose acetate and cellulose nitrate material (Millipore Corp.). The sterile solution was aseptically treated with 200 iu/ml benzylpenicillin (sodium) B.P., 200 μg/ml streptomycin sulphate B.P. (both products from Glaxo) and 100 u/ml nystatin B.P. (Mycostatin, E.R. Squibb & Sons Ltd., Twickenham, Middlesex, England).

(ii) For the establishment and maintenance of monolayer cultures

MEM with Earle's salts buffered with 25 mM HEPES (Cat. No. 041-2360) was supplemented with 20%, heat-inactivated foetal calf serum 2 mM/ml L-glutamine and received 100 iu/ml benzylpenicillin (sodium) B.P. and 100 μg/ml streptomycin sulphate B.P. as for the complete RPMI 1640.

3.5 Separation of PBL

Methods for the isolation of PBL and Theileria sporozoites were modified after Brown (1979a). PBL were separated from blood with ACD as an anticoagulant, or defibrinated, as described above. The blood was aseptically divided equally into two sterile 50-ml graduated conical centrifuge tubes which were then covered with screw-on caps and centrifuged at 2,250 xg for 30 minutes at 5°C (3,000 rpm MSE Mistral 2L; MSE Scientific Instruments, Manor Royal, Crawley,

Sussex, England) to obtain buffy coat. The supernatant plasma was discarded with a 10-ml graduated pipette until 1-2 ml remained above the buffy coat layer. The buffy coat cells were recovered in about 3 ml and diluted in 9 ml phosphate buffered saline, PBS, pH 7.3 (Dulbecco A, without Ca^{++} and Mg^{++}; Oxoid Ltd., Wade Road, Basingstoke, Hampshire, RG24 OPW). Ten to 12 ml of the diluted buffy coat suspension were carefully layered onto 8 ml Ficoll/sodium diatrizoate gradient, Sp. gr. 1.077 (Ficoll-paque; Pharmacia-Fine Chemicals AB, Uppsala, Sweden; each 100 ml of solution contains: 5.7 g ficoll 400, 9.0 g sodium diatrizoate and edetate calcium diasodium) in a disposable universal bottle (Sterilin Ltd., Teddington, Middlesex, England) and the interface subjected to 670 xg for 40 minutes at 20°C (2,000 rpm MSE Mistral 2L). After discarding the supernate, the interface cells were recovered in a total volume of 5 ml and washed twice in PBS, pH 7.3 (20 ml) at 275 xg (1,450 rpm MSE Minor) for 10 minutes, then five minutes. The cell pellet was resuspended in 10 ml complete RPMI 1640 and the concentration adjusted to 8×10^6 cells/ml.

3.6 Ticks

Adult ticks (*Hyalomma anatolicum anatolicum* and *Rhipicephalus appendiculatus*) infected with *Theileria* parasites were maintained and provided by Dr. Alan Walker of the entomology section, Centre for Tropical Veterinary Medicine (CTVM), University of Edinburgh.

3.7 Theileria parasites

3.7.1 Theileria annulata (Ankara)

The Ankara strain of Theileria annulata (Schein, Buscher and Friedhoff, 1975) was originally sent to the CTVM in a heavily infected batch of Hyalomma anatolicum excavatum ticks by Professor E. Schein, Berlin. The parasite was isolated from a naturally infected cow in the vicinity of Ankara, Turkey. Following a single inoculation into a calf, this strain of Theileria annulata was serially passaged, in Berlin, through 12 generations exclusively in the laboratory strain of H. anatolicum excavatum. At the CTVM the parasite has been maintained in H. anatolicum anatolicum.

3.7.2 T. annulata (Hissar)

The Hissar strain of T. annulata (Gill, Kaur and Bhattacharyulu, 1974) was originally sent to the CTVM in infected H. anatolicum anatolicum by Dr. Y. Bhattacharyulu of Ludhiana. Fed and unfed male and female adult H. detritum were collected off cattle from various areas of Hissar, India, during June to August, 1973 and reared in the insectory. Different active stages of the tick were allowed to engorge on fully susceptible calves. Non-infected larvae and nymphs were allowed to feed on the infected calves and when replete, were collected and allowed to moult to nymphs and adults respectively. The emerged nymphs and adults were released on susceptible calves. Nymphs from colonies of H. anatolicum anatolicum were engorged on the calves when T. annulata infection was fully developed. This virulent strain of T. annulata collected from Hissar was designated as "Hissar" strain, hence T. annulata (Hissar).

3.7.3 T. parva (Muguga)

The Muguga strain of T. parva (Brocklesby, Barnett and Scott, 1961) was sent to the CTVM from Muguga, Kenya, in infected Rhipicephalus appendiculatus ticks kindly provided by Dr. T.T. Dolan. The parasite has been maintained since isolation by repeated passages by R. appendiculatus ticks fed on cattle and is regarded as a typical strain of T. parva. The strain was also brought as the E174 T. parva-transformed lymphoblastoid cell line isolated by C.G.D. Brown.

3.8 Preparation of Theileria annulata sporozoite suspensions for infecting cells

Adult H. anatolicum anatolicum ticks infected with either the Ankara (Schein et al., 1975) or Hissar (Gill et al., 1974) strains of T. annulata and fed on rabbit ears for three days to allow maturation of sporozoites were surface sterilised by three different sequential treatments: (a) three times in 1% benzalkonium chloride (Roccal antiseptic, Winthrop Laboratories, Surrey, England), (b) three times in 70% (v/v) ethanol, (c) four times in Eagle's MEM with Hanks' salts, containing 200 iu/ml benzylpenicillin (sodium) 200 µg/ml streptomycin sulphate and 100 u/ml nystatin. The medium in the final wash was discarded and 2.5 ml of MEM/BPA containing benzyl penicillin, streptomycin sulphate and nystatin as detailed above were added and the ticks transferred to a sterile mortar. Forty ticks were ground up thoroughly with a pestle (4 rounds), each time alternately adding and recovering 2.5 ml of MEM/BPA above so that, finally 10 ml of sterile ground up tick suspension with a

concentration equivalent to four ticks/ml were obtained. The suspension was centrifuged at 100 xg for five minutes at 20°C (1000 rpm MSE Minor) and the supernate (GUTS) containing sporozoites aseptically recovered into a 20 ml sterile universal bottle. Further removal of debris was done by passing the GUTS through a sterile 25 mm Swinnex filter holder, with a type AP25 depth filter and 8 µm MF filter of cellulose acetate and cellulose nitrate material (Millipore Corp). Fifty µl filtered GUTS were centrifuged at 700 rpm for ten minutes in a centrifuge (Cytospin Centrifuge; Shandon Southern Instruments Ltd., Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR) onto microscope slides and smears stained with Giemsa (Shute, 1966), prepared as detailed below, to check for the presence, the morphology and the concentration of sporozoites in the supernate.

3.9 Preparation of Merck's Giemsa stain

Ten g Giemsa powder (E. Merck, D61 Darmstadt, Germany) were weighed out and poured into a large pyrex glass mortar (Gallenkamp and Company Ltd., P.O. Box 290, Technico House, Christopher Street, London, England), some glycerol (Analar grade; BDH Chemicals Ltd.) added and the mixture ground up using a large pyrex glass pestle (Gallenkamp and Co. Ltd.). The suspension was transferred into a two-litre, conical pyrex flask (Gallenkamp and Co. Ltd.). The rest of the glycerol was used to gradually wash off the contents of the mortar, all the washings being transferred to the conical flask. A total of 540 ml of glycerol was used. The flask, with its contents, was placed in a water bath previously heated to, and

maintained at, 60°C, with intermittent shaking. The suspension was then cooled to room temperature, 840 ml methyl alcohol (Solvent; BDH Chemicals Ltd.) and the mixture shaken gently but thoroughly. The flask was transferred onto a monostir magnetic stirrer (Gallenkamp and Co. Ltd.) and stirred overnight following which 2.76 g (0.2 g/100 ml) of Azur II were added to the suspension and the stirring continued for another 24-48 hours. The preparation was finally filtered through cellulose filter paper No. 4 (Whatman Laboratory Products Ltd., Springfield Mill, Maidstone, Kent, ME14 2LE) and the filtrate stored in 500-ml aliquots in dark bottles ready for use with Giemsa buffer prepared by dissolving buffer tablets "GURR" 65500 (BDH) in deionised distilled water (one tablet per litre of deionised distilled water - pH 7.2).

3.10 Preparation of T. annulata sporozoites for electron microscopy

3.10.1 From GUTS

T. annulata sporozoites in GUTS were purified and concentrated by a method based on discontinuous density gradient using Percoll* (Pharmacia-fine Chemicals AB) as a gradient medium (Ulmer and Flad, 1969) and modified after Walker and McKellar (1983). A stock solution of iso-osmotic Percoll (SIP) was made and diluted to the desired lower densities as described below: to 5 ml Hanks' balanced salt solution without Mg^{++} or Ca^{++} (x10) (HBSS; Gibco-Europe) were added 0.001 g $NaHCO_3$ and 0.5 g bovine plasma albumin (BPA, Armour Fraction V). The concentrated HBSS/BPA was sterilised by filtration through a sterile 25 mm Swinnex filter with type AP25 depth filter and a 0.22 µm MF filter as described above. Iso-osmotic

*Percoll consists of colloidal silica particles of 15-30 nm diameter which have been coated with polyvinyl pyrrolidone (PVP)

stock solution of Percoll (SIP) was made by mixing nine parts (v/v) of sterile Percoll with one part of the HBSS/BPA above. The pH of the mixture was adjusted to 7.2 by a dropwise addition of sterile 1NHCl and constant stirring. The final density of SIP in HBSS/BPA medium was 1.121 g/ml, whereas the HBSS/BPA medium was 1.010 g/ml. Dilution of SIP to the desired lower densities was undertaken by adding specific quantities of HBSS/BPA medium as tabled below.

Volume of SIP (ml)	+ Volume of HBSS/BPA (ml)	= Step density (g/ml)
7.6	2.4	1.095
5.4	4.6	1.070
1.8	3.2	1.050
0.9	4.1	1.030

Following the dilution of the SIP, the purification of GUTS was carried out. A non-siliconised, graduated, 40-ml, glass centrifuge tube was placed in a slant, test tube rack at 45°. Density steps were carefully and slowly dispensed down the side of the centrifuge tube in order of decreasing density starting with the 1.095 step, using a 5-ml syringe fitted with a 25G x 1 inch needle. Whereas 5 ml, each, of the 1.095 and 1.070 density steps were dispensed, 2-ml aliquots of the 1.050 and 1.030 steps were introduced into the tube in the slant rack. Eight ml GUTS were finally layered slowly and carefully onto the 1.030 step layer and the 1.095/1.070 interface subjected to 3,250 xg for 60 minutes at 5°C (4,500 rpm, MSE Chilspin). The tube was then put in an upright position in a rack and the gradient material removed until about 2 ml remained above the 1.095/1.070 interface. The interface material, consisting exclusively of mature T. annulata sporozoites, was carefully sucked off using a

screw-operated syringe and centrifuged at 1,500 xg for 30 minutes at 5°C (3,000 rpm, MSE Chilspin) to remove Percoll. The pellet of sporozoites was resuspended in 8 ml Eagle's MEM with Hanks' salt containing 1% bovine plasma albumin, 200 iu/ml benzylpenicillin, 200 µg/ml streptomycin sulphate and 100 u/ml nystatin. Fifty-µl aliquots of the purified sporozoite suspension were used to prepare cytocentrifuge smears (Cytospin centrifuge; Shandon Southern Instruments Ltd.) which were stained with Giemsa stain (Shute, 1966). The remainder of the material was pelleted at 1,500 xg for 15 minutes at 5°C (3,000 rpm, MSE Chilspin) and processed for electron microscopy as described below.

3.10.2 In whole salivary glands

Ten adult H. anatolicum anatolicum ticks infected with T. annulata parasites and fed on rabbit ears for three days to allow maturation of sporozoites were washed in a domestic sieve under a jet of cold tap water and individually picked with forceps to eliminate contaminating debris such as hair from rabbits. The ticks were embedded with the ventral surfaces in wax (melting point 57°C) melted with a moderately heated spatula. After the wax solidified, they were immediately covered with several drops of ice-cold PBS, pH 7.3 (Dulbecco A, Oxoid Ltd.) and the dorsal integument dissected off under a stereomicroscope using a scalpel blade No. 11 (Swann Morton Ltd., Sheffield, England) and two pairs of watch-maker's forceps, Nos. 4 and 5A. Heavily infected salivary glands were gently teased out, removed and immersed in ice-cold, 2.5% gluteraldehyde (TAAB Laboratories, Reading, England) in 0.1M sodium cacodylate (TAAB) buffer and processed for electron microscopy as described below.

3.11 Establishment of cultures

While T. parva-lymphoid cultures were established in 2 cm^2 wells of tissue culture plates (Costar, Broadway, Cambridge, Massachusetts) in which monolayer cultures of bison lung fibroblastic cell line, IMR31, (Flow Laboratories, Victoria Park, Heatherhouse Road, Irvine, KA12 8NB, Scotland), had been established 2-3 days previously, T. annulata cultures did not require feeder layers and were set up without any. In both cases, 0.25 ml PBL suspension (2×10^6 cells) and 0.25 ml GUTS were seeded into each culture well. The cultures were immediately put into a humidified plastic box, gassed with 5% CO_2/95% air and incubated at 37°C.

3.12 Maintenance of cultures

Each culture well received 0.5 ml complete RPMI 1640 on Day 1 and 1 ml on Day 3. On Day 6, 1 ml supernatant medium was removed from each well and replaced with fresh complete RPMI 1640. Every three days, 1 ml of culture medium was removed and replaced with an equal volume of fresh complete RMPI 1640.

Assessment of cultures for cell morphology and characteristics, and percentage of infected cells (PIC) were undertaken during each medium change. For PIC determinations, 50-µl aliquots of thoroughly mixed cell suspensions were used for preparing cytocentrifuge smears which were stained with Giemsa stain. When PIC, determined by counting 200 lymphoid cells, reached 75% cultures were transferred and maintained in disposable plastic flasks of 25 cm^2 surface area (Nunc, Roskilde, Denmark) according to the technique described by Brown (1979a). They were initially passaged at a ratio of 1:1

(i.e. 5 ml cell suspension from the original flask transferred to a fresh flask containing 5 ml fresh complete RPMI 1640 pre-warmed to 37°C), then passaged at 1:4 and later at 1:9 three times weekly.

3.13 Cell count determinations

3.13.1 Freshly prepared PBL

Trypan blue for determining the viability of cells was dissolved in 0.9% NaCl (Boyum, 1968) and used as a 0.2% solution. Equal parts of the dye and cell suspension were mixed and allowed to stand for five minutes. The mixture was diluted at 1:20 with 0.5% glacial acetic acid solution in a white-cell-diluting pipette and counted in a haemocytometer (New Improved Neubauer; Macfarlane and Robson Ltd., Hedgefield House, Blaydon-on-Tyne, Tyne-and-Wear, U.K.) as described by Schalm, Jain and Carroll, 1975) using a x10 objective on an Ortholux microscope (Ernst Leitz, Ernst Leitz Strasse, Wetzlar, W. Germany). Unstained leucocytes in four corner primary squares were counted and multiplied by a factor of 50 to obtain the number of viable cells in 1 µl of suspension. Since the cell suspension had originally been diluted 1:1 with trypan blue, the number of cells obtained above was further multiplied by two.

3.13.2 Culture suspensions

Cell counts were carried out using a haemocytometer (New Improved Neubauer) according to Schalm *et al.* (1975) and a x10 objective on an Ortholux microscope (Ernst Leitz). 0.1 ml of a thoroughly mixed culture suspension was mixed with 0.1 ml of 0.2%

trypan blue in a bijou bottle and allowed to stand for five minutes. The mixture was then pipetted up and down and introduced into a haemocytometer chamber. Unstained cells in four corner primary squares were counted, divided by four and the mean multiplied by 10^4* to obtain the number of viable cells/ml. Since the cell suspension had originally been diluted 1:1 with trypan blue solution, the number of cells obtained above was further multiplied by two. The total number of cells in a culture vessel was obtained by multiplying the number of cells/ml by the volume of the culture suspension.

3.14 Light microscopy

Examination of Giemsa-stained smears, autoradiography preparations, as well as determination of cell numbers were undertaken using either the Ortholux or the Dialux 20 microscopes (Ernst Leitz).

3.15 Transmission electron microscopy

Suspensions of cells for electron microscopy were centrifuged at 275 xg for ten minutes at 20°C (1,450 rpm, MSE Minor). After discarding the supernatant growth medium the pellets were resuspended in 5 ml serum-free RPMI 1640 (Moore et al., 1967) warmed to 37°C and spun at 275 xg for another ten minutes at 20°C as above. The resultant pellets were fixed overnight at 4°C in 2.5% glutaraldehyde (TAAB Laboratories) in 0.1M sodium cacodylate (TAAB Laboratories) buffer, pH 7.4. Whole tick salivary glands obtained as

*The depth of the chamber is 0.1 mm. The four corner primary squares are each 1 mm x 1 mm. The volume is, therefore, 0.1 mm^3. Since 1000 mm^3 equals 1 ml, the number of cells in 0.1 mm^3 is multiplied by 10,000 to obtain the number of cells in 1 ml of culture suspension.

described above were transferred directly into the 2.5% glutaraldehyde. Samples were washed in 3 x 10 minute changes of 0.1M cacodylate buffer, pH 7.4, at 4°C and then fixed for two hours in 1% osmium tetroxide, OsO_4, (Johnson-Matthey Chemicals Ltd., 74 Hatton Garden, London, England) in 0.1M cacodylate buffer, pH 7.4, at 4°C. Osmication was followed by 3 x 10 minute changes in 0.1M cacodylate buffer, pH 7.4 at 4°C. Dehydration of samples was undertaken in increasing concentrations of ethanol as follows: 2 x 15 minutes in 10%, 2 x 15 minutes in 50%, 2 x 15 minutes in 90% and finally 2 x 15 minutes in absolute ethanol. They were then cleared twice, 15 minutes each time, in propylene oxide (Fisons, Loughborough, England). Propylene oxide was discarded and replaced by 1:1 mixture of propylene oxide and araldite CY212 (Agar Aids, 66a Cambridge Road, Stanstead, Essex, England) for one hour after which the samples were left in pure araldite for 24 hours to allow maximum infiltration of the tissues with the embedding medium. The pellets and salivary gland specimens were eventually placed in fresh araldite with hardener HY964 and accelerator BDMA (both products of Agar Aids) and polymerised at 60°C for two days. Sections were cut with LKB glass knives (LKB-Produkter AB, S161 25 Bromma, Sweden) on a Cambridge-Huxley Mark II Ultramicrotome (Kent Cambridge Medical Co. Ltd., Histon Road, Cambridge, England) and mounted on 200-mesh copper grids (Gilder Ltd., Sinclair, West Kensington, London, England). Grids were stained as follows: they were placed in a saturated solution of uranyl acetate (Analar grade; BDH Chemicals Ltd.), as described by Watson (1958), in 50% ethanol for 15 minutes in the dark and rinsed in 10% ethanol. The grids were rinsed in distilled water,

then placed in lead citrate (EMScope Laboratories Ltd., Kingsworth Industrial Estate, Wolton Road, Ashford, Kent, England) solution (Reynolds, 1963) for five minutes in the dark and rinsed in distilled water. The grids were picked up with forceps and very gently touched to a cellulose filter paper (Whatman Ltd.) on the slide without sections. They were finally air-dried and examined on a Philips 400 electron microscope (Philips Nederland B.V., Hoofdgroep PPS, Postbus 90050, 5600PB, Eindhoven).

3.16 Autoradiography

Thymidine has been shown to be a specific precursor of DNA (Friedkin, Tilson and Roberts, 1956) and the low mean β-energy released during the decay of tritium, (^{3}H), facilitates excellent resolution on autoradiographs (Taylor, Woods and Hughes, 1957). The incorporation of (^{3}H) thymidine into the host cell and parasite nuclei was consequently employed as an indicator of DNA synthesis. Sixteen hours before pulse-labelling, cell lines were subcultured in a total of 5 ml complete RPMI 1640. Whereas T. parva (Muguga) cell line was passaged at 2 x 10^{5} cells/ml, T. annulata (Ankara) and T. annulata (Hissar) cell lines were each subcultured at 10^{5} cells/ml of complete RPMI 1640. At 16 hours after passages were made, each cell line received 10 μCi/ml (6-^{3}H) thymidine (Amersham International, White Lion Road, Amersham, Buckinghamshire, England; specific activity, 23 Ci/mmol). The dose level of 10 μCi/ml had been used successfully previously with T. parva-infected cell lines (Irvin, Ocama and Spooner, 1982). After a 15-minute exposure to tritiated thymidine, cell suspensions were transferred to 20 ml universal bottles (Sterilin Ltd., Teddington, Middlesex, England)

and centrifuged at 275 xg for ten minutes at 20°C (1,450 rpm, MSE Minor). The pellets were resuspended in fresh complete RPMI 1640, gently mixed by pipetting and centrifuged again at 275 xg for five minutes. The total time was considered as a 30-minute exposure. The supernates were discarded in special bottles and later disposed of in a "radioactive waste" sink. The pellets were resuspended in fresh complete RPMI 1640 and 50-µl aliquots obtained for cytocentrifuge smears. Cell suspensions were centrifuged once more at 275 xg for five minutes as above, the pellets resuspended in fresh complete RPMI 1640, gassed with 5% CO_2/95% air and incubated at 37°C. In the case of T. parva (Muguga), T. annulata (Ankara) and T. annulata (Hissar) cell lines, samples for smears were obtained immediately after the 30-minute exposure to tritiated thymidine followed by a couple of samples at four hours and eight hours. Four replicate cytocentrifuge preparations of each cell line were examined at each sampling. Smears were rapidly air-dried, fixed in technical methanol and coated by dipping (Joftes and Warren, 1955) in Ilford L4 nuclear emulsion (Polysciences Inc., Paul Valley Industrial Park, Warrington, Philadelphia 18976, USA). Coating and development of smears were undertaken in a darkroom using a method modified after Williams (1977). A fresh stock of emulsion was removed from a refrigerator and allowed to warm to darkroom temperature (18°C) at a constant relative humidity of 60-70% for about 45-60 minutes. During this period the safelight socket was fitted with a 15-watt bulb and the unit covered with Kodak Wratten Series 2 filter (Kodak Ltd., Box 33, Swallowdale Lane, Hemel Hempstead, Hertfordshire, UK), and the room was checked for light-tightness. A water bath, fitted

with a thermometer and containing a recommended quantity of water, was switched on and the temperature allowed to stabilise at 44-45°C. A specially designed perspex container, to hold the emulsion, 78 mm long x 55 mm broad x 40 mm deep, was placed in the water bath, submerged to $\frac{3}{4}$ its depth and firmly positioned. A 150-ml beaker was weighed on a sliding weight balance and 5 g added to its weight on the scale. With only the safelight on, and the darkroom otherwise light-tight, 5 g emulsion gel were weighed out into the 150-ml beaker which was then transferred to the water bath for ten minutes after adding 5 ml deionised, distilled water. The molten emulsion was carefully poured into the pre-warmed perspex container, the slides dipped and withdrawn vertically and slowly (about ten seconds standard time for each slide). Coated slides were hung up away from the safelight using clothes pegs, dried for 45-60 minutes in front of a fan and stored in 8 x 10 cm, black, plastic slide boxes (A.R. Horwell Ltd., 2 Grangeway, Kilburn Road, London, England), containing fresh silica gel in velin tissue, sealed with black scotch electrical tape No. 33 (3M UK Ltd., 3M House, Wigmore Street, London, England). Securely closed stock of emulsion and sealed boxes were transferred to a refrigerator at 4°C. Batches of coated slides were stored for five or ten days prior to development thus exposing the emulsion to β-particles emitted by decaying tritium. One hour prior to development, slides were removed from the refrigerator and allowed to warm up to darkroom temperature (18°C) in their sealed boxes with silica gel. They were developed for five minutes in glass staining dishes using freshly prepared, doubly filtered D19 developer (Kodak Ltd., Box 33, Swallowdale Lane, Hemel Hempstead, Hertfordshire)

in a darkroom lit with a safelight fitted with a 15-watt bulb and an Ilford 904F filter (Polysciences Inc.). The developer was then poured off and slides rinsed in 100 ml deionised distilled water at 18°C for 30 seconds.

One hundred ml Kodak rapid fixer (Kodak Ltd.) were poured on and the slides fixed for ten minutes without agitation. The fixer was poured off and slides rinsed in deionised distilled water briefly, then in slowly running tap water for at least 30 minutes following which they were dried in front of a fan for 45-60 minutes and stained for five minutes in 10% Giemsa buffered to pH 7.2. DNA synthesis in both the schizont and the host cell was assessed by examining 400 cells/smear for tritiated thymidine incorporation at each time period. Photography was undertaken using an automatic Orthomat camera (Ernst Leitz).

3.17 Buffers

3.17.1 Phosphate buffered saline (Dulbecco "A"; Oxoid Ltd.)

The Oxoid Dulbecco "A" solution corresponds to the original formulation of Dulbecco and Vogt (1954) but modified by omitting Ca^{++} and Mg^{++}. The solution was prepared using Dulbecco "A" tablets (Oxoid; Code BR 14a) of the following formula:

Formula	grams/litre
Sodium chloride	8.0
Potassium chloride	0.2
Disodium hydrogen phosphate	1.15
Potassium dihydrogen phosphate	0.2

Ten tablets were dissolved in one litre of deionised distilled water

and autoclaved at 15 lb/sq inch for ten minutes (115°C). The resultant pH was 7.3.

3.17.2 Cacodylate buffer

Two stock solutions A and B were prepared. Stock solution A, 0.2M sodium cacodylate, was prepared by dissolving 4.28 g sodium cacodylate powder (molecular weight, 214; TAAB) in 100 ml of deionised distilled water. Stock solution B, 0.2N HCl, was prepared by pipetting 1.7 ml concentrated hydrochloric acid (BDH) in 100 ml deionised distilled water. Cacodylate buffer, pH 7.4 was made by mixing 25 ml of solution A with 1.4 ml of solution B and making the total volume up to 100 ml with deionised distilled water.

3.17.3 Sodium phosphate buffer

Two stock solutions were prepared. Stock solution A, 0.2M sodium dihydrogen orthophosphate, was prepared by dissolving 3.12 g sodium dihydrogen orthophosphate (molecular weight, 156; Analar grade; BDH) in 100 ml deionised distilled water. Solution B, 0.2M disodium hydrogen orthophosphate, was prepared by dissolving 2.83 g disodium hydrogen orthophosphate (molecular weight, 142; Analar grade; BDH) in 100 ml distilled water. Sodium phosphate buffer of pH 7.2 was prepared by mixing 14.0 ml solution A with 36.0 ml solution B and making the total volume up to 100 ml with deionised distilled water.

3.17.4 Tris-HCl buffer

Two stock solutions A and B were prepared. Solution A, 0.2M Tris, was prepared by dissolving 2.42 g Tris (hydroxymethyl)

methylamine (molecular weight, 121.1; general purpose reagent; BDH) in 100 ml deionised distilled water. Stock solution B, 0.2 NHCl, was prepared as above by dissolving 1.7 ml concentrated hydrochloric acid in 100 ml deionised distilled water. Twenty-five ml of solution A was mixed with 13.4 ml solution B and made up to 100 ml with distilled water in order to obtain a solution of pH 8.0.

3.17.5 Tris-maleate buffer

The buffer was prepared by mixing two stock solutions A and B. Solution A, 0.2M Tris acid maleate, comprised 2.42 Tris (hydroxymethyl) methylamine above and 2.32 g maleic acid (molecular weight, 116; Analar grade; BDH) in 100 ml deionised distilled water. Solution B, 0.2N sodium hydroxide, was prepared by dissolving 0.8 g sodium hydroxide (molecular weight, 40; Analar grade; BDH) in 100 ml distilled water. A mixture of 25 ml of A and 3.5 ml of B made up to 100 ml with distilled water gave a solution of pH 5.2.

3.17.6 Sorensen's phosphate buffer

The buffer was prepared by mixing two solutions A and B. While solution A was prepared by dissolving 11.876 g disodium hydrogen orthophosphate above in a litre of deionised distilled water, solution B was prepared by dissolving 9.08 g potassium dihydrogen orthophosphate (molecular weight, 119; Analar grade; BDH) in a litre of deionised distilled water. Sorensen's buffer, pH 7.6 was obtained by mixing 88.5 ml of solution A with 11.5 ml solution B.

3.18 Statistical methods

All the statistical methods used in this thesis are conventional as described by Sokal and Rohlf (1981).

CHAPTER FOUR

INVASIVE BEHAVIOUR AND INTRACELLULAR DEVELOPMENT OF T. ANNULATA SPOROZOITES IN PBL

4.8 Encounter of, and interaction with, host lymphocyte lysosomal system by the developing T. annulata sporozoites

4.8.1 Introduction

4.8.2 Materials and methods

4.8.3 Results

4.9 Discussion

CHAPTER FOUR

INVASIVE BEHAVIOUR AND INTRACELLULAR DEVELOPMENT OF T. ANNULATA SPOROZOITES IN PBL

4.1 Introduction

The entry of parasites into cells is a crucial step in the pathogenesis of an infection. Precise knowledge of the initial events that characterise host target cell-parasite interaction is, consequently, important in the development of protective measures against disease entities. In animal diseases caused by protozoan parasites of the genus Theileria, little is known about the mechanism by which sporozoites, the stage in the salivary glands of the arthropod vector ticks infective for their various bovine, ovine and caprine vertebrate hosts, enter the target cells. There is a similar paucity of information on the morphological changes they undergo before developing into the multinucleate, intralymphocytic stage, the schizont.

As a prerequisite to the interiorisation of intracellular parasites, such as Theileria species, contact must first be made with the host cell of predilection. The contact may either be made haphazardly as in the case of flagellates (Miller and Twohy, 1967; Akiyama and Haight, 1971; Lewis, 1974; Alexander, 1975) or in a particular orientation as in the case of merozoites of Babesia (Rudzinska, Trager, Lewengrub and Gubert, 1976) and Plasmodium (Miller, Dvorak, Shiroishi and Durocher, 1973; Dvorak, Miller, Whitehouse and Shiroishi, 1975), and sporozoites of Lankesteria culicis (Sheffield, Garnham and Shiroishi, 1971) in which the anterior end of the parasites usually attach to the host cell membrane.

The latter case would incriminate specific reciprocal recognition sites on the parasite limiting membrane and host cell plasmalemmal surfaces.

Interiorisation may be achieved by disrupting the host cell plasmalemmal membrane as do the members of the microsporidian genus Nosema (Weidner, 1972; 1976), by active invasion and invagination of the host cell plasmalemma without disrupting its continuity e.g. Selenidium hollandei (Metais and Schrevel, 1974), Eimeria magna (Jensen and Hammond, 1975; Jensen and Edgar, 1976) and mammalian malaria parasites, Plasmodium berghei and P. knowlesi (Danforth, Aikawa, Cochraine and Nussenzweig, 1980) or mainly by the phagocytic activity of the host cell as for amastigotes of Leishmania donovani (Chang and Dwyer, 1978; Pearson, Romito, Symes and Harcus, 1981) and Leishmania mexicana promastigotes and Trypanosoma cruzi trypomastigotes and promastigotes (Alexander, 1975; Nogueira and Cohn, 1976).

Once an intracellular parasite is interiorised, its survival depends on its ability to circumvent lysosomal activity and destruction. This may be achieved either by withstanding the toxic substances and digestive enzymes delivered to the phagocytic vacuole as in Leishmania donovani (Chang and Dwyer, 1978) and Leishmania mexicana (Alexander and Vickerman, 1975) or by inhibiting phagosome-lysosomal fusion as in the case of Toxoplasma gondii (Jones and Hirsch, 1972), Encephalitozoon cuniculi (Weidner, 1975) and Chlamydia psittaci (Eissenberg and Wyrick, 1981).

In an electron microscopic study of the entry of Theileria parva into bovine lymphocytes, Fawcett et al. (1982b) believed that

sporozoites entered in any orientation, that the investing membrane acquired during entry of the sporozoites disappeared 24 hours after interiorisation and permitted the parasite to evade destruction by the phagolysosomal system, and that even dead and degenerate sporozoites invaded lymphocytes. This chapter examines structural characteristics of T. annulata sporozoites, their interaction with PBL and their intracellular development involving the encounter and interaction with the host lymphocyte lysosomal system.

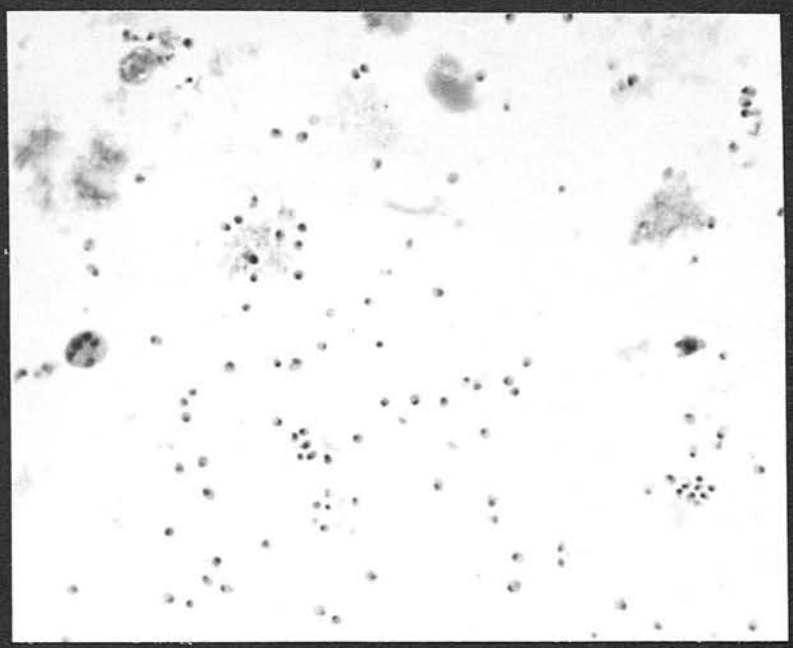
4.2 Morphological characteristics of mature T. annulata sporozoites and PBL

4.2.1 T. annulata sporozoites

Giemsa-stained cytocentrifuge smears of T. annulata sporozoite suspensions prepared as a GUTS filtrate (Figure 1) or purified using Percoll gradient medium (Figure 2) illustrated the ovoid shape of this stage of the parasite and the eccentric displacement of its dark-staining nucleus. Whereas Figure 2 showed a morphological uniform population, the GUTS material (Figure 1) demonstrated phases in the maturation of T. annulata sporozoites ranging from a young sporoblast containing several nuclei to mature ones from which sporozoites were budding off. The ultrastructure of the parasite within whole salivary gland acini of 3-day fed Hyalomma anatolicum anatolicum showed the presence of both immature and the final sporozoite-forming stages (Figure 3). The immature parasite appeared as an extensively branched, anastomosing, multinucleate syncytium which interdigitated with, and filled, the host cytoplasm (Figure 4). Parasite units with one to several nuclei, one or more mitochondria,

Figure 1 A Giemsa-stained, cytocentrifuge smear of T. annulata sporozoite suspension prepared as GUTS filtrate from 3-day fed, heavily infected H. anatolicum anatolicum ticks. Several phases of sporozoite maturation are illustrated including a young, multinucleate sporoblast, a mature sporoblast unit from which sporozoites are budding off, numerous ovoid, uninucleate sporozoites, and residual bodies.
Magnification, x 1,280

Figure 2 A Giemsa-stained, cytocentrifuge smear of T. annulata sporozoite suspension prepared as GUTS filtrate from 3-day fed, heavily infected H. anatolicum anatolicum ticks and purified using Percoll gradient medium. A morphologically uniform population of ovoid, uninucleate sporozoites is illustrated and represents a band of GUTS fraction recovered at the interface between step densities 1.070/1.095 g/ml.
Magnification, x 1, 280



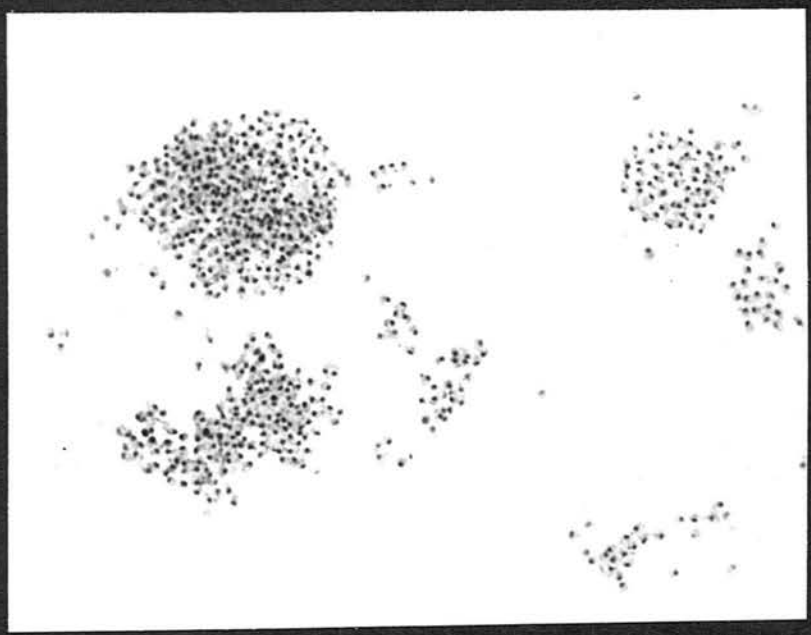


Figure 3 An electron micrograph of two different phases of T. annulata sporozoite maturation in two adjacent cells within a salivary gland acinus of a 3-day fed infective H. anatolicum anatolicum. The cell on the left-hand side of the micrograph contains an immature parasite growing as a syncytium and filling the host cell cytoplasm, while the one on the right shows the final sporozoite-forming phase with many parasite units liberating sporozoites.
Magnification, x 6,827

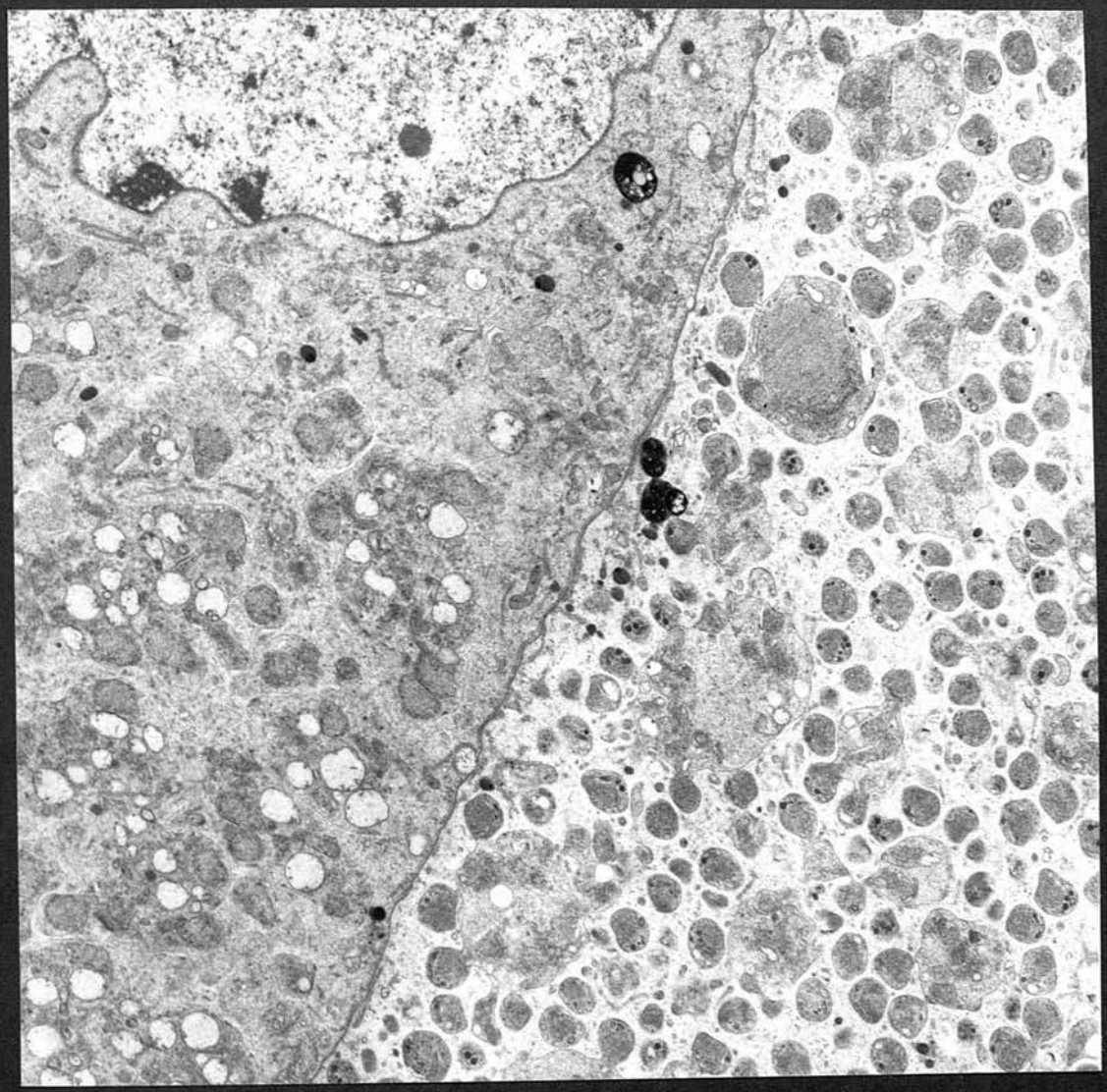
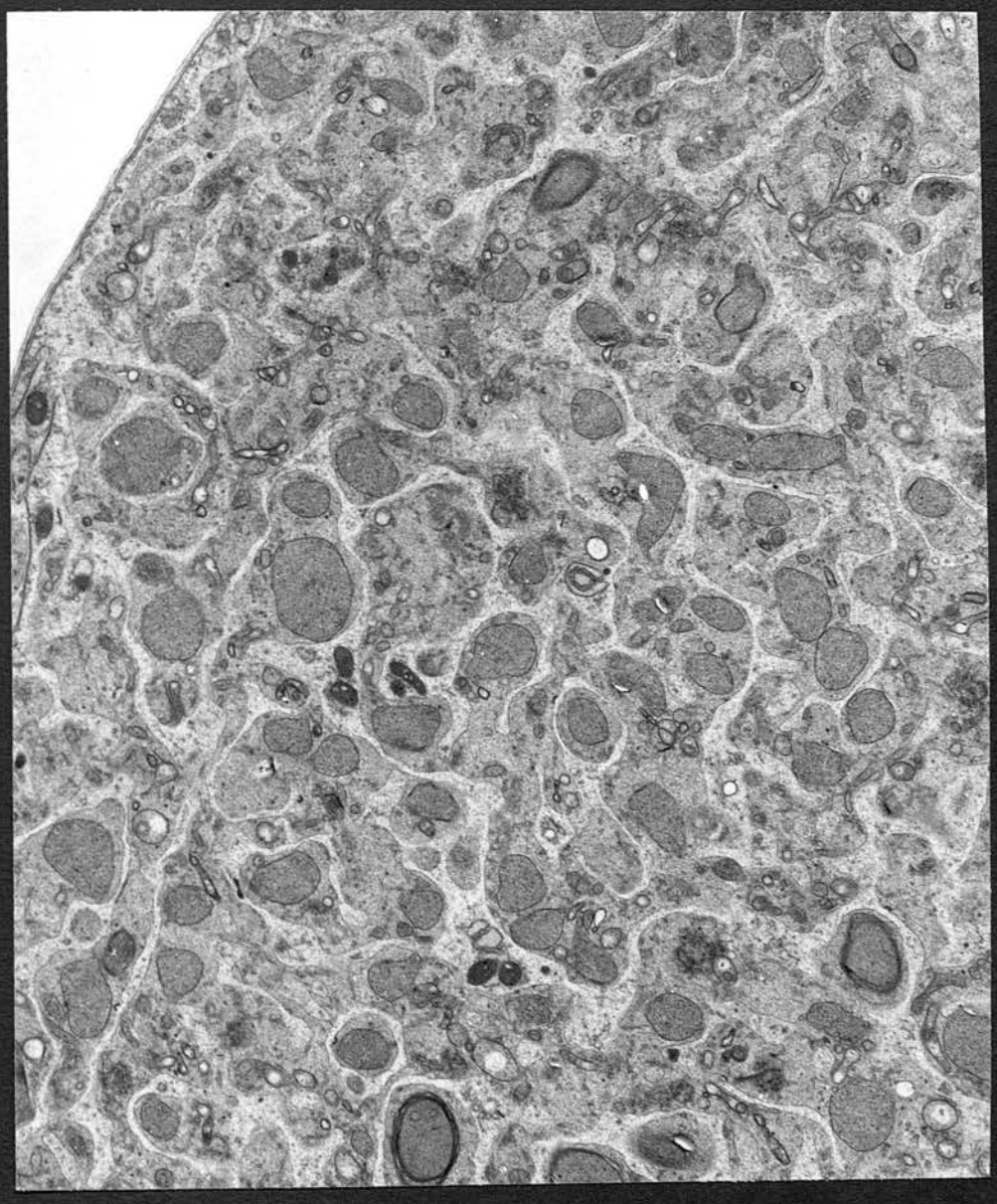


Figure 4 An immature T. annulata parasite within a salivary gland acinar cell of a 3-day fed infective H. anatolicum anatolicum. The parasite appears as an extensively branched, anastomosing, multinucleate syncytium which interdigitates with, and fills, the host cell cytoplasm.
Magnification, x 6,878



but devoid of rhoptries (Figures 5 and 6) appeared to derive from the aforementioned syncytium. Massive schizogonous production of T. annulata sporozoites appeared to occur from the units (Figure 7). Infective sporozoites, fixed in situ within salivary gland acini or processed from either GUTS or Percoll gradient medium, manifested similar ultrastructural features (Figure 8). They were usually ovoid, measured on average about 0.9 μm long, 0.8 μm broad and were surrounded by a thin, limiting unit membrane, the pellicle, 80-100Å thick. They did not possess a surface coat and had no additional supporting membranes on the inner aspect of the pellicle apart from the polar ring. Situated at the basal end of the sporozoite was a round to ovoid nucleus encased in a nuclear envelope composed of two membranes, each 75Å thick. The outer nuclear membrane was patchily covered by ribonucleoprotein particles, 150-250Å diameter. The nucleoplasm contained no nucleoli and showed no distinct organisation of chromatin fibrils. It consisted of uniformly distributed closely packed ribonucleoprotein-like particles, 75-200Å diameter. The ground substance of the cytoplasm contained numerous, loosely scattered, electron-dense ribosomal particles, 150-250Å diameter. Mitochondria were acristate, tubular and were enclosed by two membranes. The inner membrane was thicker and measured 100Å while the relatively thinner outer membrane was 60Å thick. The intramembranous space was lucent and measured about 40Å in thickness. The shape of the mitochondria varied from round, curved, dumb-bell, elongate or resembled a pear while the diameter of the luminal space ranged from 0.02 to 0.33 μm. The rhoptries, densely osmiophilic organelles, were anterior to the nucleus and assumed different shapes

Figure 5 An electron micrograph of a salivary gland acinar cell of a 3-day fed H. anatolicum anatolicum infected with T. annulata. Parasite units devoid of rhoptries appear to budd off from branches of the sporoblast syncytium. What appears to be a residual body is manifest in the lower right-hand corner of the micrograph.
Magnification, x 15,494

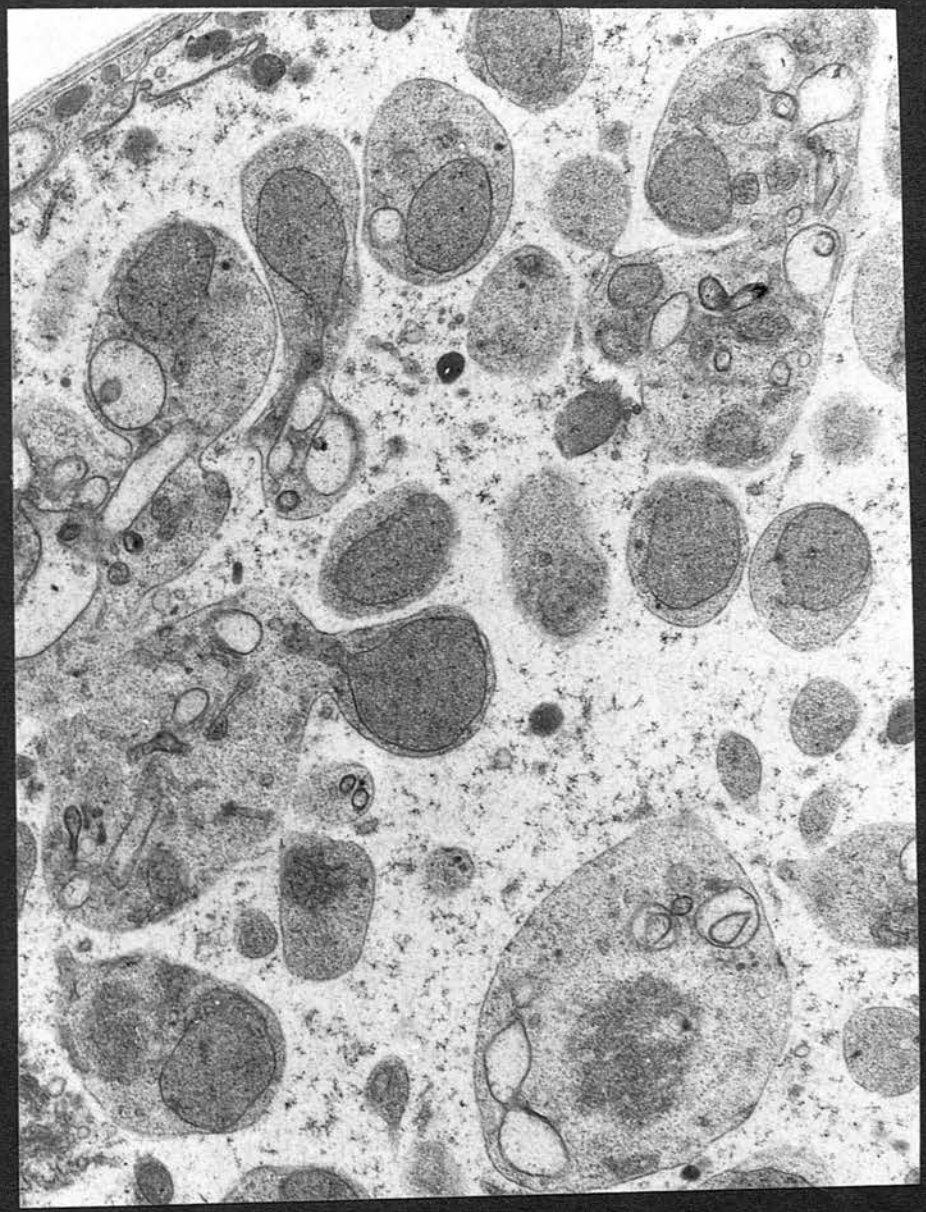


Figure 6 Similar to Figure 5. More rhoptry-lacking T. annulata sporoblast units formed from the syncytium.
Magnification, x 15,024

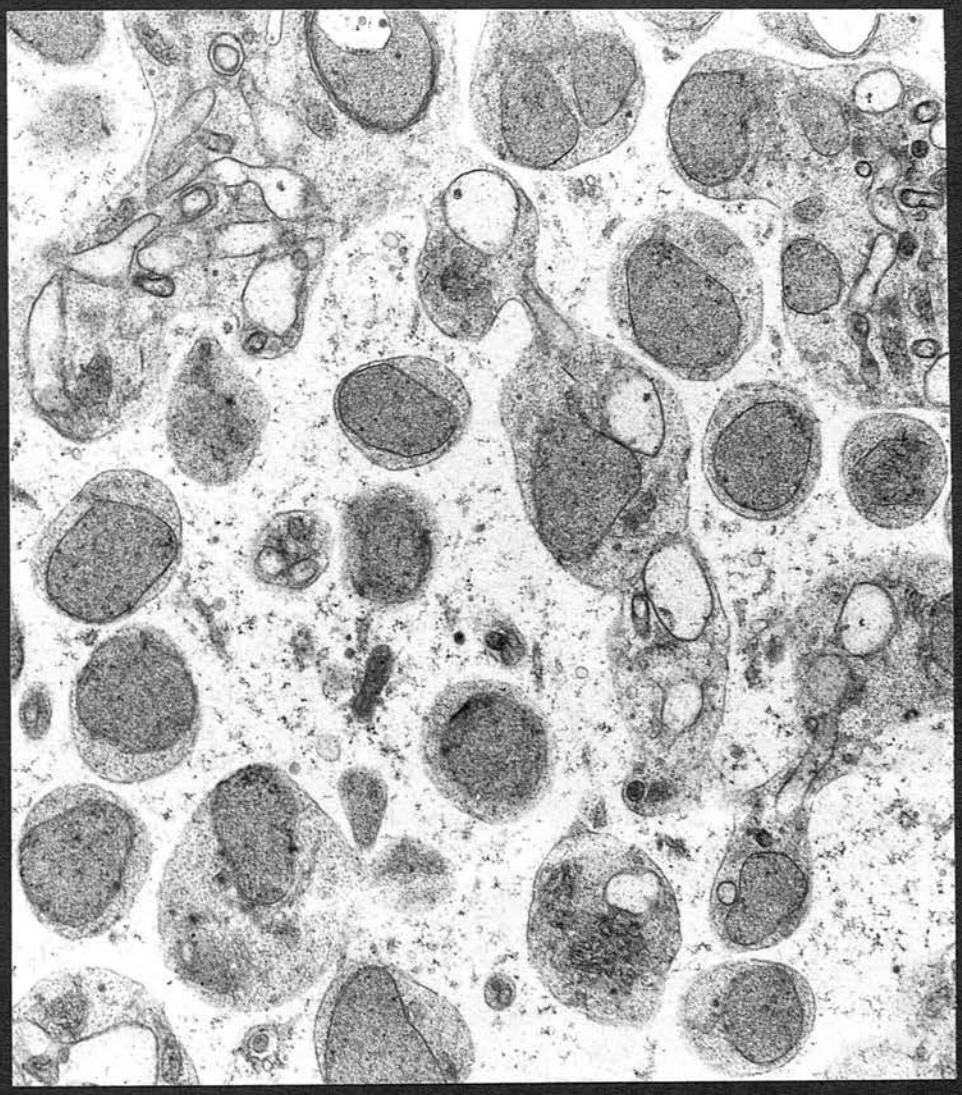


Figure 7 The final phase of T. annulata sporozoite development, within an acinar cell of a 3-day fed H. anatolicum anatolicum, showing massive schizogonous production of infective sporozoites from units of a sporoblast. Magnification, x 7,011

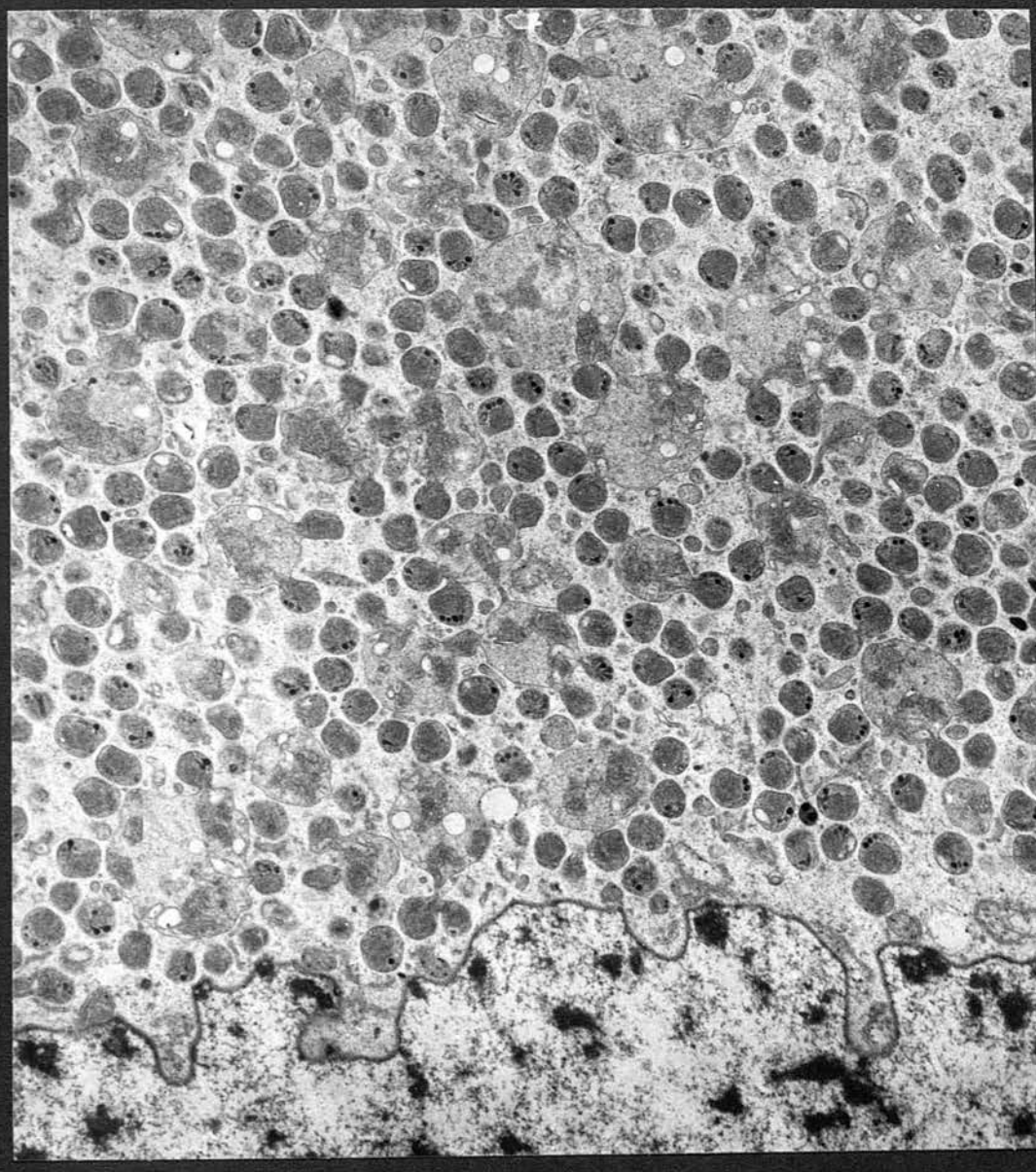
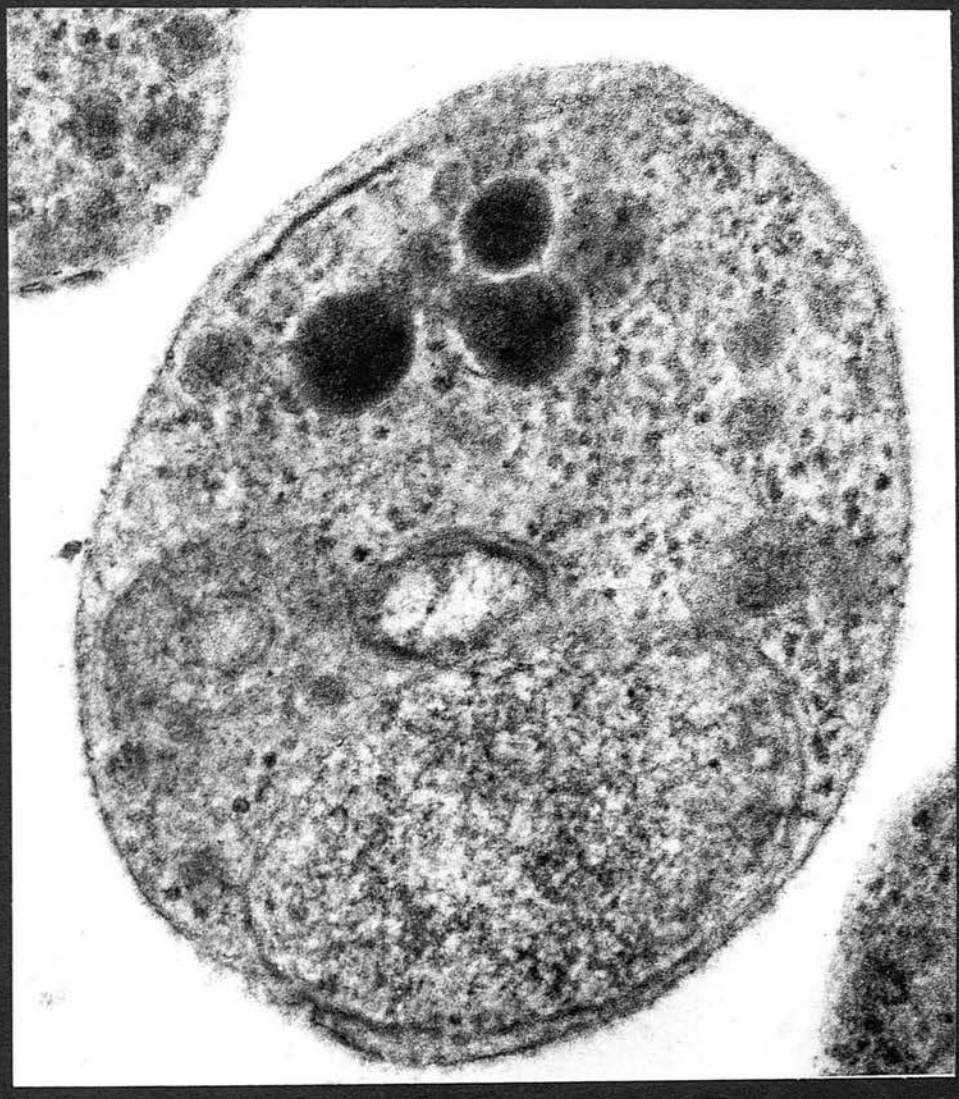


Figure 8 T. annulata sporozoite showing fine structural characteristics. The apical complex consists of several rhoptries and a polar ring. The sporozoite is surrounded by a unit membrane, the pellicle, and contains in the ground substance of its cytoplasm, numerous ribosomes, and acristate mitochondria. The nucleus is eccentric and non-chromocentric.
Magnification, x 196,778



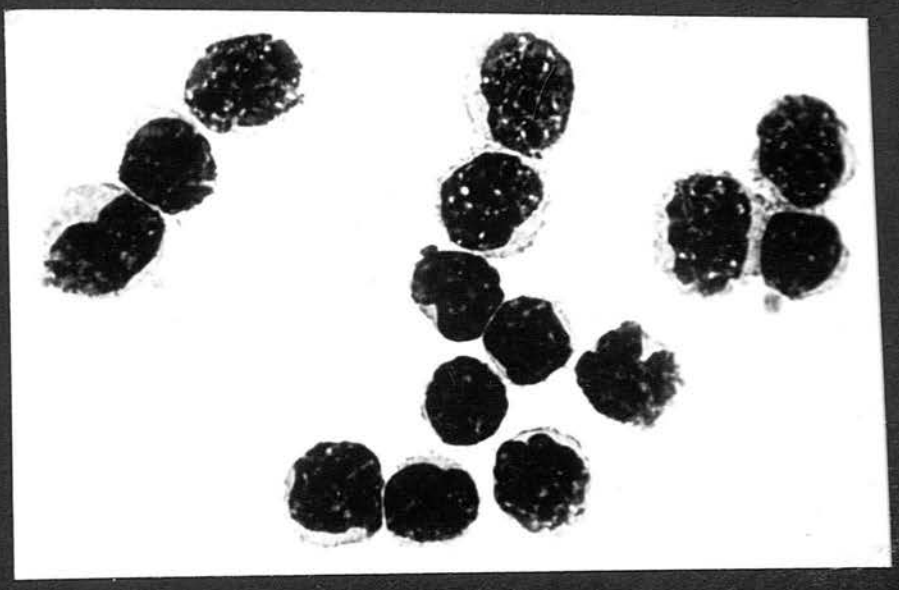
and sizes depending on the plane of section. Several rhoptries together with the polar ring represented the apical complex in T. annulata sporozoites.

4.2.2 PBL

Figures 9 and 10 represent light and electron micrographs of bovine peripheral blood lymphocytes separated from defibrinated blood by the Ficoll/sodium diatrizoate gradient technique as detailed in Chapter 3. The lymphocytes generally manifested some degree of heterogeneity in their ultrastructural characteristics. Common features (Figure 11) included (1) a large irregularly shaped or deeply indented, vesicular nucleus containing (a) large masses of densely clumped heterochromatin, the chromocentres commonly associated with the inner aspect of the inner nuclear membrane, (b) reticulating, fine heterochromatic threads, the chromonemata, and (c) patches of the much more electron-lucent euchromatin, (d) one or two indistinct, spherular nucleoli associated, in some cells, with small clumps of heterochromatin at the periphery; (2) a generally rudimentary, but occasionally well developed Golgi complex; (3) a few well developed cristate mitochondria which showed a tendency to be grouped on the nuclear "hof" pole of the cell, the centrosphere; (4) occasional single strands of rough endoplasmic reticulum; (5) numerous free ribosomes; (6) an inconsistent number and distribution of surface microvilli; and (7) one or two centrioles.

Figure 9 A Giemsa-stained, cytocentrifuge smear of bovine peripheral blood lymphocytes separated from defibrinated blood by the Ficoll/sodium diatrizoate gradient technique.
Magnification, x 1,280

Figure 10 An electron micrograph of PBL separated by the Ficoll/sodium diatrizoate gradient technique. The cell suspension consists almost entirely of lymphocytes.
Magnification, x 9,200



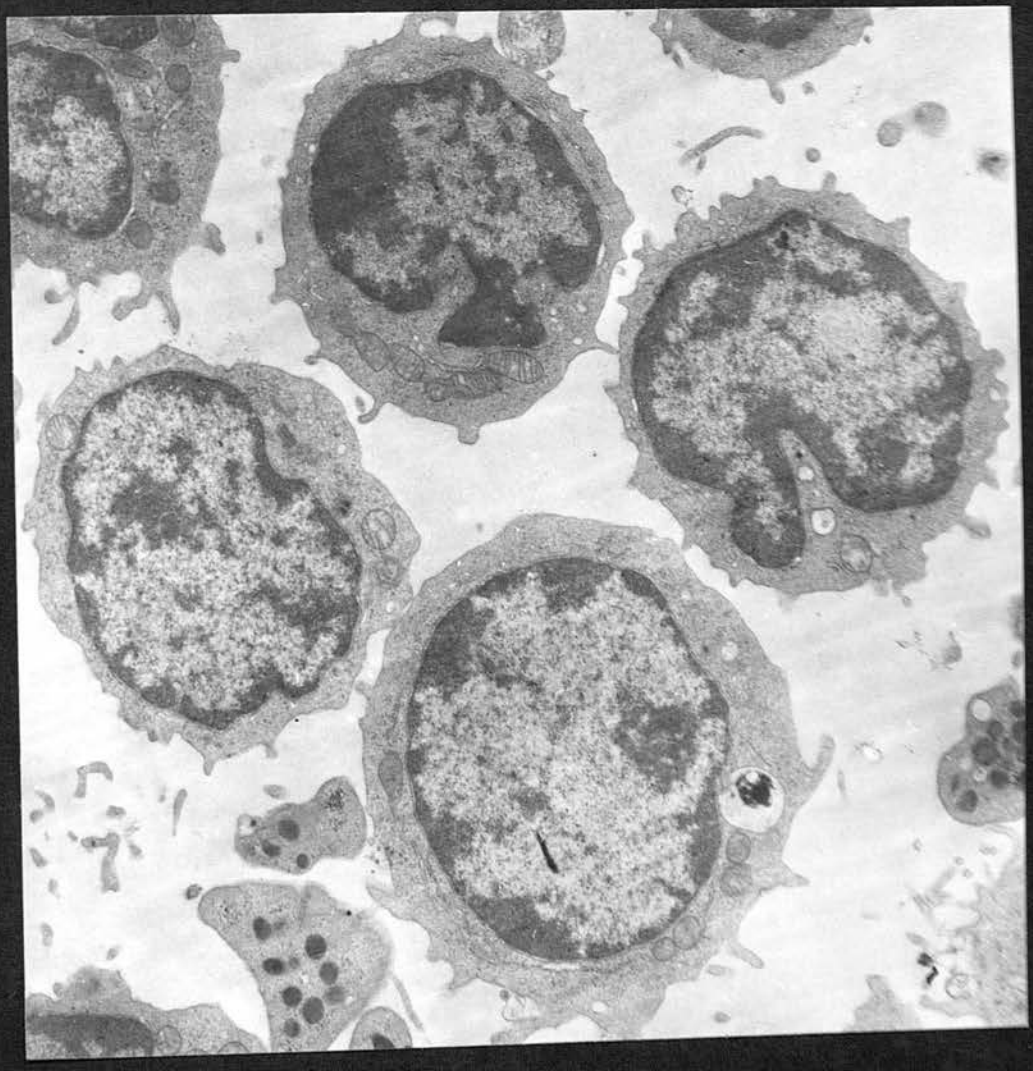
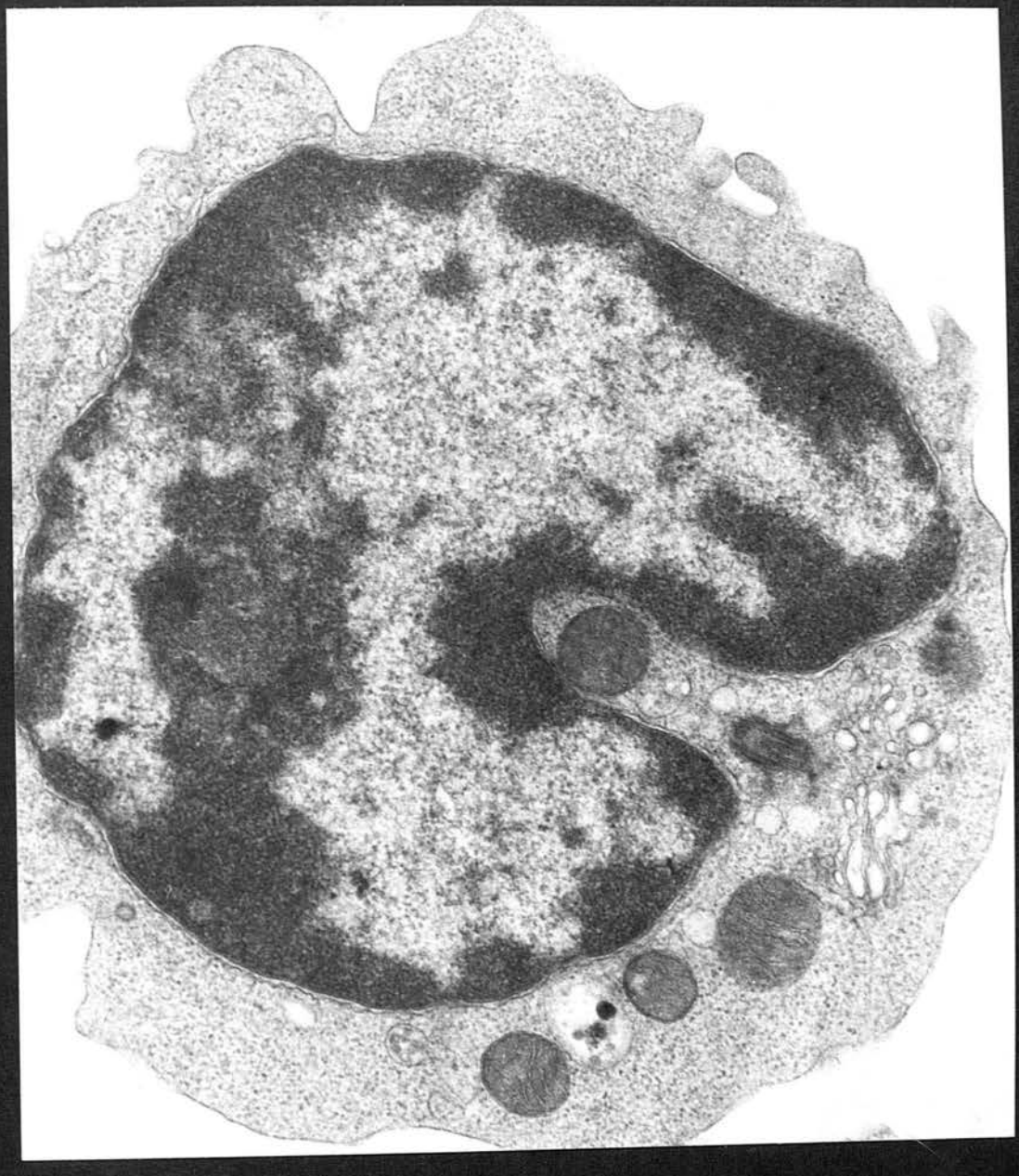


Figure 11 A non-infected bovine peripheral blood lymphocyte. A deeply indented vesicular nucleus shows chromocentres and chromonemata and patches of electron-lucent euchromatin. A ring-like nucleolus is associated with clumps of perinucleolar heterochromatin. Golgi complex, a centriole with pericentriolar satellite, and mitochondria occupy the nuclear "hof" pole of the lymphocyte. Occasional strands of rough endoplasmic reticulum, and numerous ribosomes are seen in the cytoplasm. Magnification, x 31,935



4.3 Invasion of PBL by T. annulata sporozoites and early intracellular developmental events

4.3.1 Interaction patterns and temporal distribution of T. annulata sporozoites in PBL

Giemsa-stained cytocentrifuge smears made five minutes after mixing and incubating T. annulata sporozoites with PBL at 37°C demonstrated interiorised parasites within some cells. The determination of the percentage of infected cells out of 200 lymphoid cells per slide in eight slides at 15 minutes, 3 hours and 18 hours revealed means of 5.9 ± 1.07%, 12.5 ± 1.62% and 14.4 ± 1.14% respectively. Two groups of susceptible lymphocytes were observed (Figure 12): one group in which sporozoite entry localised on one pole of the plasmalemma while the other subpopulation of cells had sporozoites distributed uniformly around the plasmalemmal surface.

The temporal distribution of T. annulata sporozoites within susceptible PBL, separated from defibrinated blood, was investigated in eight replicate cultures established in 2 cm^2 wells of tissue culture cluster plates as described under general materials and methods and sampled for cytocentrifuge smears at 15 minutes, 3 hours and 18 hours. In each replicate smear, the number of sporozoites within each cell was determined in a total of 40 infected cells. The mean number of T. annulata sporozoites within susceptible PBL at each time period is presented in Table 1 and detailed data in the Appendix Table 1. One way analysis of variance revealed that the differences between time periods were highly significant ($F^2_{21} = 229.85$; $P < 0.01$) and further analysis by Duncan's multiple range test showed that each time period was significantly different

Figure 12 A Giemsa-stained, cytocentrifuge smear prepared at 1 hour of incubation showing two different groups of susceptible bovine lymphocytes. A, which are invaded by T. annulata sporozoites from one pole of the plasma membrane and B, in which the penetration is uniform around the lymphocyte plasmalemmal surface.
Magnification, x 1,280

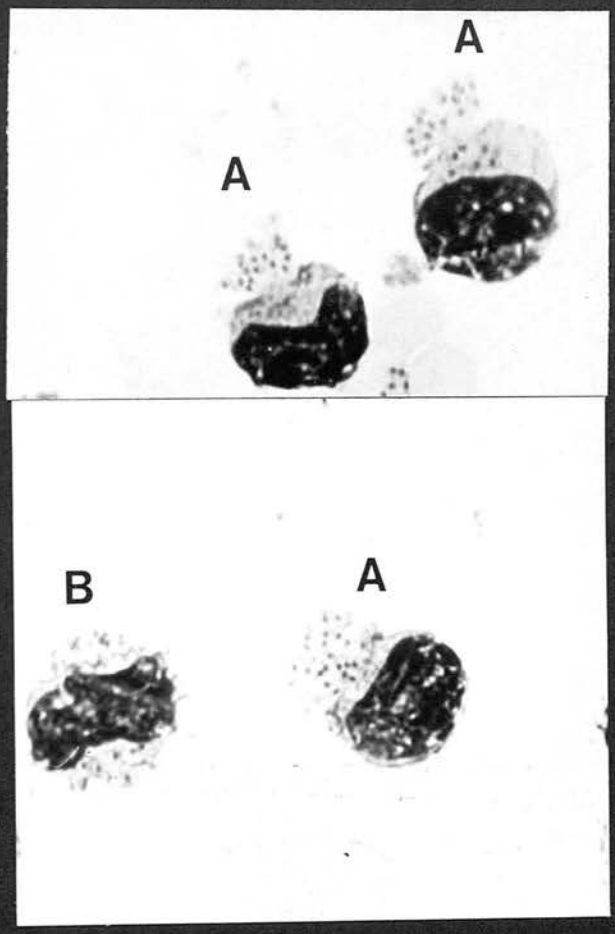

A
A
B
A

Table 1 The influence of length of incubation on the number of T. annulata sporozoites invading PBL per 40 infected cells

Time period (hours) n = 8	Means and standard deviations
0.25	85.6 ± 13.8
3	212.1 ± 25.76
18	340.4 ± 29.27

from the others (F^{1}_{21} = 397.97; $P < 0.01$). The relationship between the number of sporozoites and time periods, while it was significant, was not linear (F^{1}_{21} = 61.74; $P < 0.01$) and showed that the larger difference occurred between 0.25 hours and 3 hours.

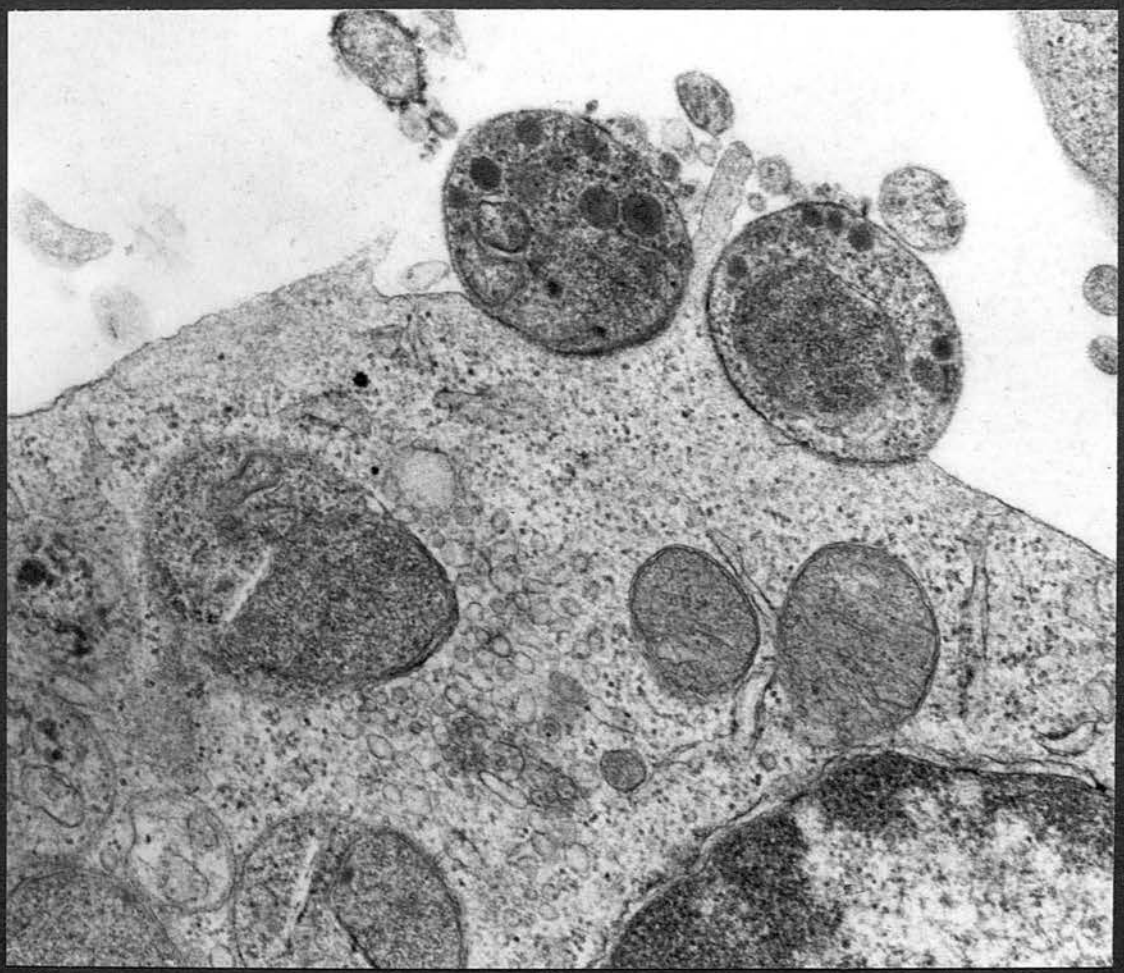
4.3.2 Sequential developmental changes

The study was undertaken on cultures established and maintained as described under the chapter on general materials and methods. Samples for both light and electron microscopy were obtained at 5, 10, 20, 30, 40 and 60 minutes, and 2, 3, 6, 18, 24 and 48 hours of incubation.

Occasionally, sporozoites in the vicinity of cells developed a pseudopod projection of the pellicle on the basal aspect subjacent to the nucleus (Figure 13). Samples fixed at five minutes of incubation (Figure 14) showed T. annulata sporozoites at various stages of invasion. At this early stage the sporozoites still possessed their characteristic ovoid shape and the specialised, densely

Figure 13 A T. annulata sporozoite processed immediately after mixing PBL with sporozoites and incubating the mixture at 37°C. The sporozoite, depicted in the vicinity of cells, has developed a pseudopod extension of its pellicle on the basal aspect subjacent to the nucleus. Magnification, x 125,000

Figure 14 An early stage of invasion illustrating multiple infection and attachment. Each of the T. annulata sporozoites has made contact with the host plasma membrane by the aspect of its pellicle adjacent to its nucleus. The apical end of the sporozoites comprising the rhoptries and mitochondria is away from, and does not come in contact with, lymphocyte plasmalemma.
Magnification, x 38,700



osmiophilic organelles, the rhoptries. The parasite consistently made contact with plasmalemma of the host lymphocyte by their basal end adjacent to the nucleus. As the invading sporozoite advanced, there was a concomitant deepening invagination of the plasmalemma of the target cell (Figure 15). The ends of the invaginated host cell membrane eventually met and fused around the sporozoite. The interiorised parasites occupied a juxtanuclear position in the lymphocytes within 30 minutes of incubation, became roundish, completely lost their rhoptries and transformed into a new stage in the life cycle of T. annulata, the trophozoite (Figure 16). The circumjacent host cell membrane which remained intact throughout the interiorisation process was seen to fragment and disappear progressively so that the trophozoite came into direct contact with the host cell cytoplasm in which state the parasite remained for the rest of its intracellular existence. The trophozoite developed increasing numbers of profiles of rough endoplasmic reticulum (Figure 17) which were in some cases continuous with the outer nuclear membrane (Figure 18) and in different sections appeared as vesicles, coils or flat, elongate cisternae. A feeding organelle, the cytostome (Figure 19), with inner and outer diameters of about 60 and 107 nm, respectively, was observed. A large number of ribosomal rosettes, the polysomes, 30-60 nm diameter developed in the ground substance of the growing trophozoites. The developing parasites were on occasions observed to be surrounded, especially around 18-24 hours, by numerous vesicular structures which appeared to be lysosomes (Figure 20). A pseudopod extension of the trophozoite pellicle, 0.5 μm long, was observed (Figure 21). Around 24 hours

Figure 15 T. annulata sporozoite shown advancing, with its basal end containing the nucleus, into a target lymphocyte as the latter's plasmalemma invaginates.
Magnification, x 39,560

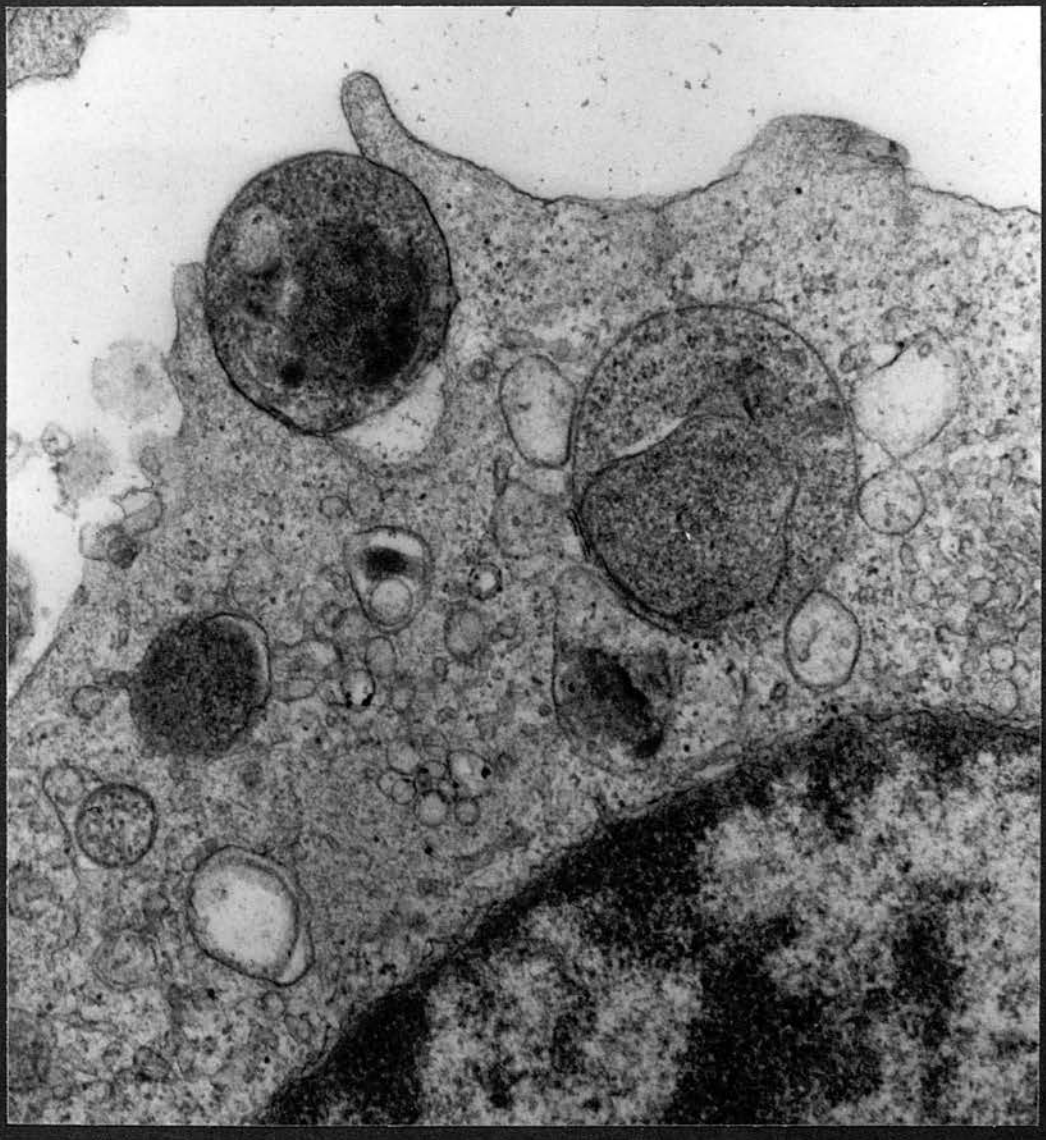


Figure 16 Specimen fixed at 30 minutes of incubation. The interiorised T. annulata sporozoite has rounded up, lost its rhoptries and transformed into a trophozoite seen closely associated with the host lymphocyte nucleus. At this stage the host cell membrane which fuses around the sporozoite during interiorisation is seen fragmenting and disappearing. The arrow depicts a fragment of the host cell membrane. The membrane has completely disappeared in the anterior aspect of the trophozoite close to the mitochondria, but is mainly intact toward the trophozoite nucleus.
Magnification, x 113,077

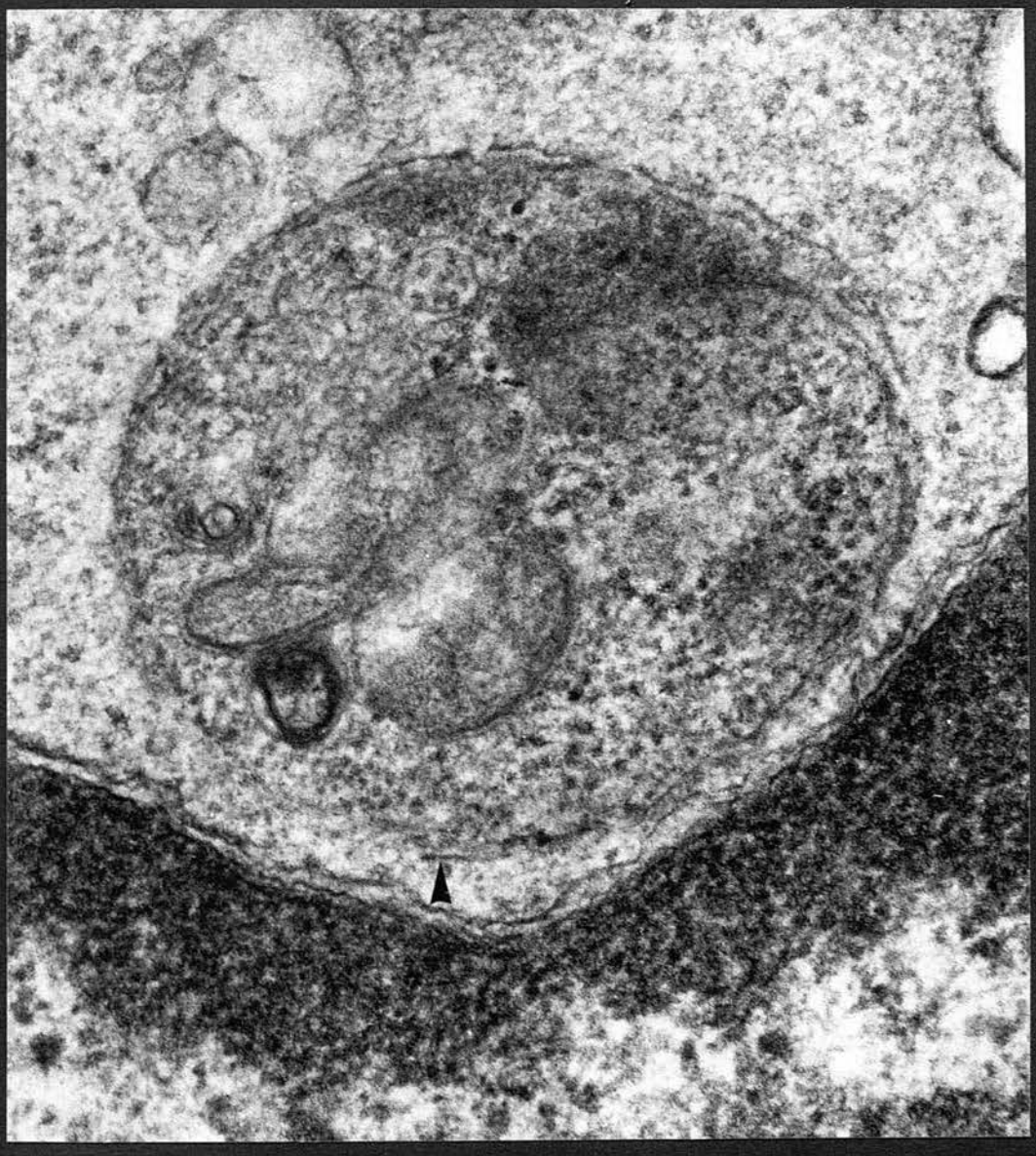


Figure 17 A T. annulata trophozoite within the cytoplasm of the lymphocyte around 18 hours of incubation. Numerous polyribosomes, well developed profiles of rough endoplasmic reticulum and acristate mitochondria.
Magnification, x 71,000

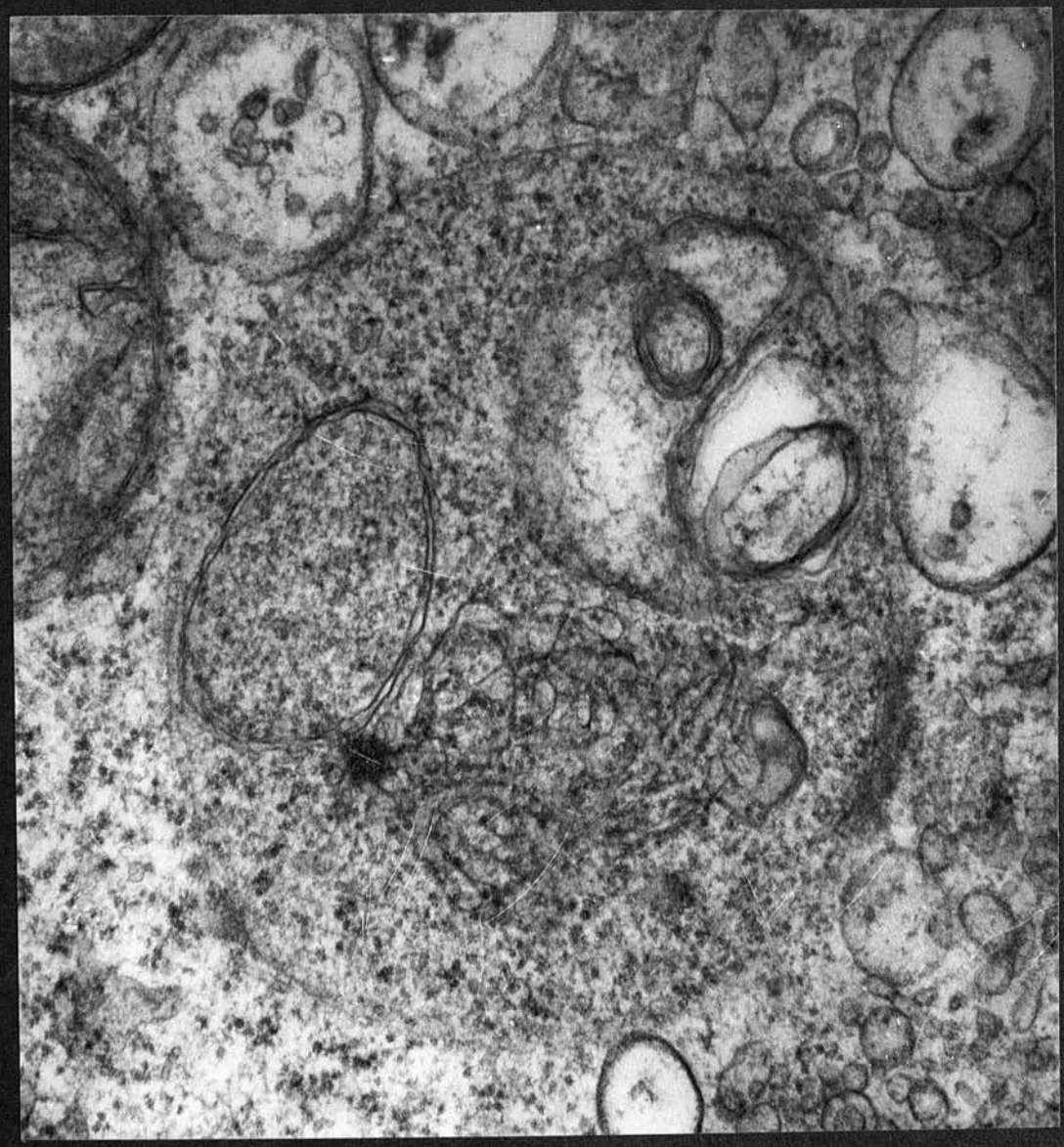


Figure 18 A fully developed T. annulata trophozoite within the cytoplasm of its host lymphocyte around 24 hours of incubation. The parasite cytoplasm contains a rich compendium of polyribosomes, a well developed rough endoplasmic reticulum which is continuous with the outer nuclear membrane and an acristate mitochondrion. A section of intranuclear spindle microtubules heralding imminent parasite nuclear division is manifest. Magnification, x 92,000

Figure 19 A T. annulata trophozoite within the cytoplasm of its host lymphocyte around 18-24 hours. A feeding organelle, the cytostome, is well developed and is seen taking in a chunk of host cell cytoplasm.
Magnification, x 118,022

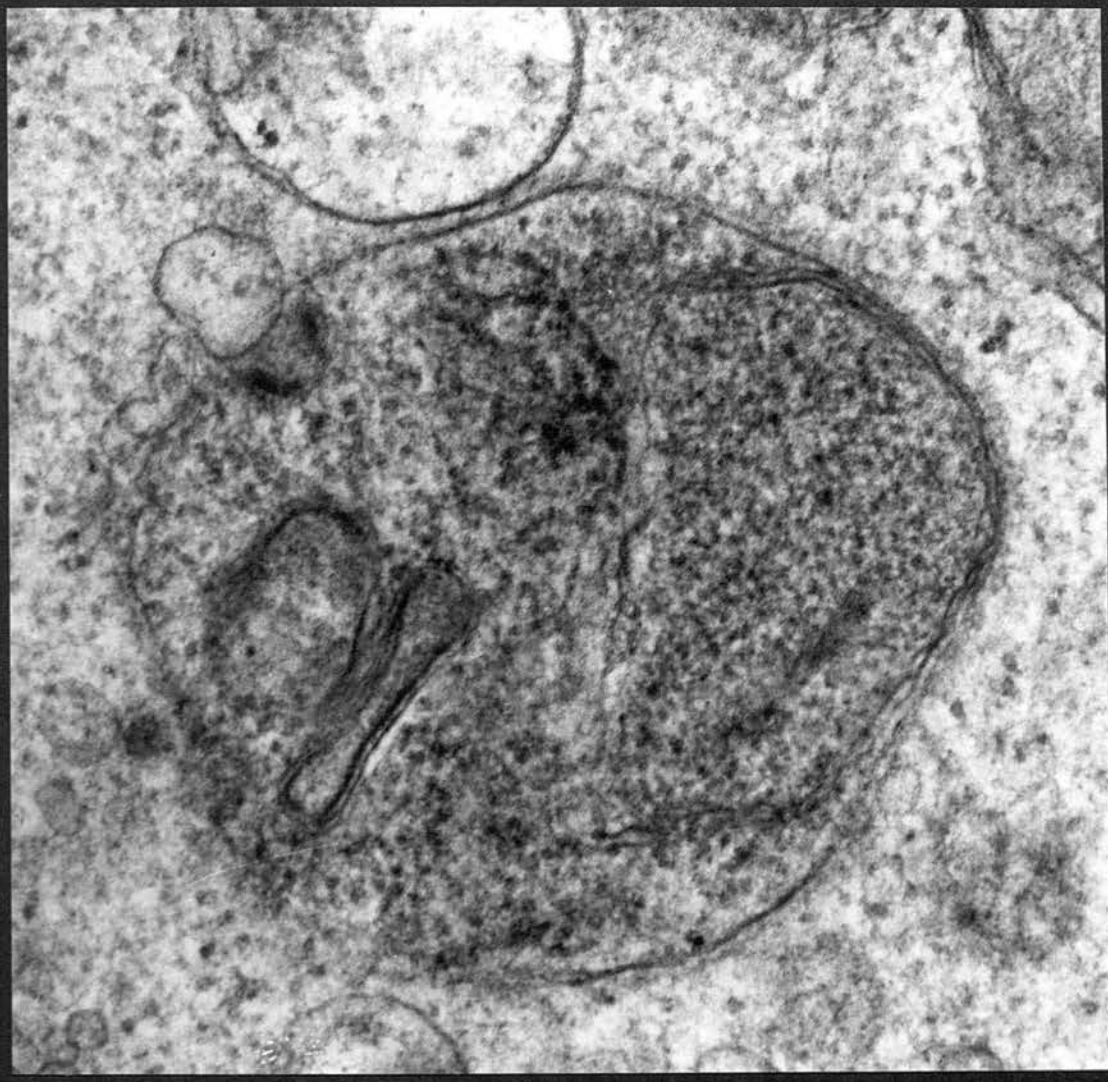


Figure 20 A T. annulata trophozoite is depicted, 18-24 hours of incubation, surrounded by numerous vesicular structures of host cell origin, presumably lysosomes. The parasite displays well developed mitochondria and profiles of rough endoplasmic reticulum, occasionally continuous with the pellicle.
Magnification, x 69,701

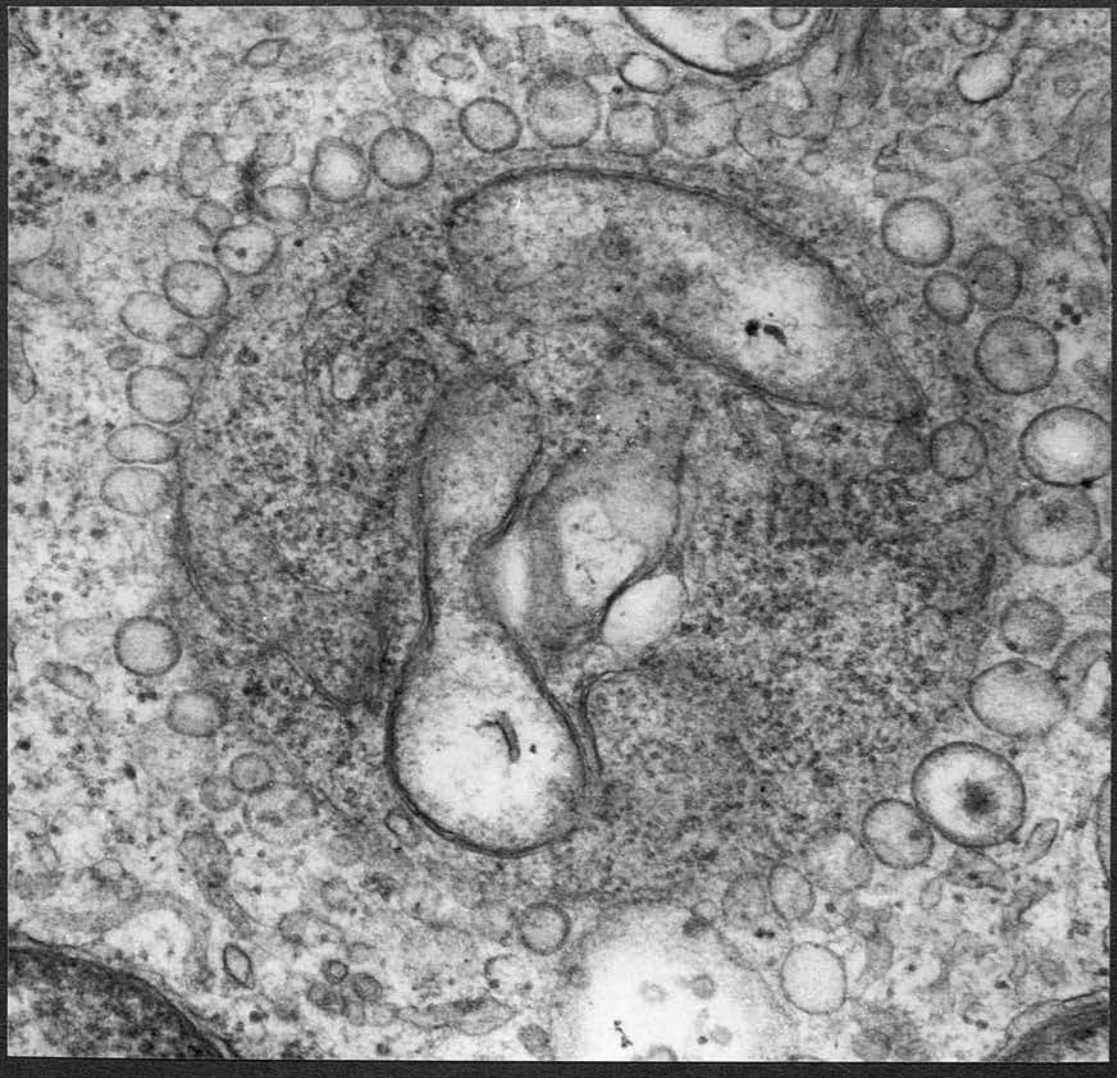


Figure 21 An electron micrograph of T. annulata trophozoite within its host lymphocyte. A pseudopod extension of the parasite pellicle is illustrated projecting into the host cytoplasm.
Magnification, x 53,472

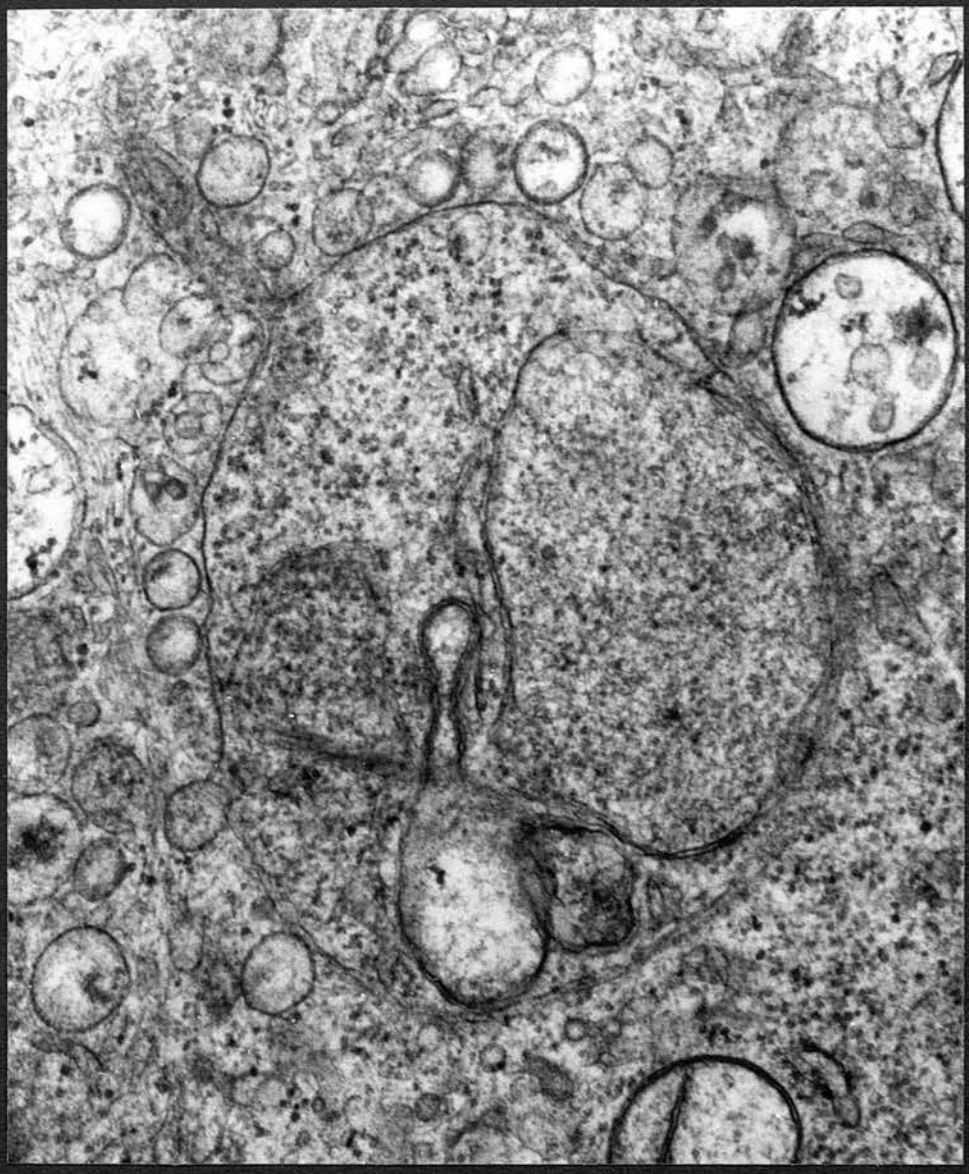


Figure 22 At 24 hours of incubation, a T. annulata trophozoite manifests well developed, anisotropic, intranuclear spindle microtubules depicting imminent division of the trophozoite nucleus to produce a schizont. Magnification, x 120,000

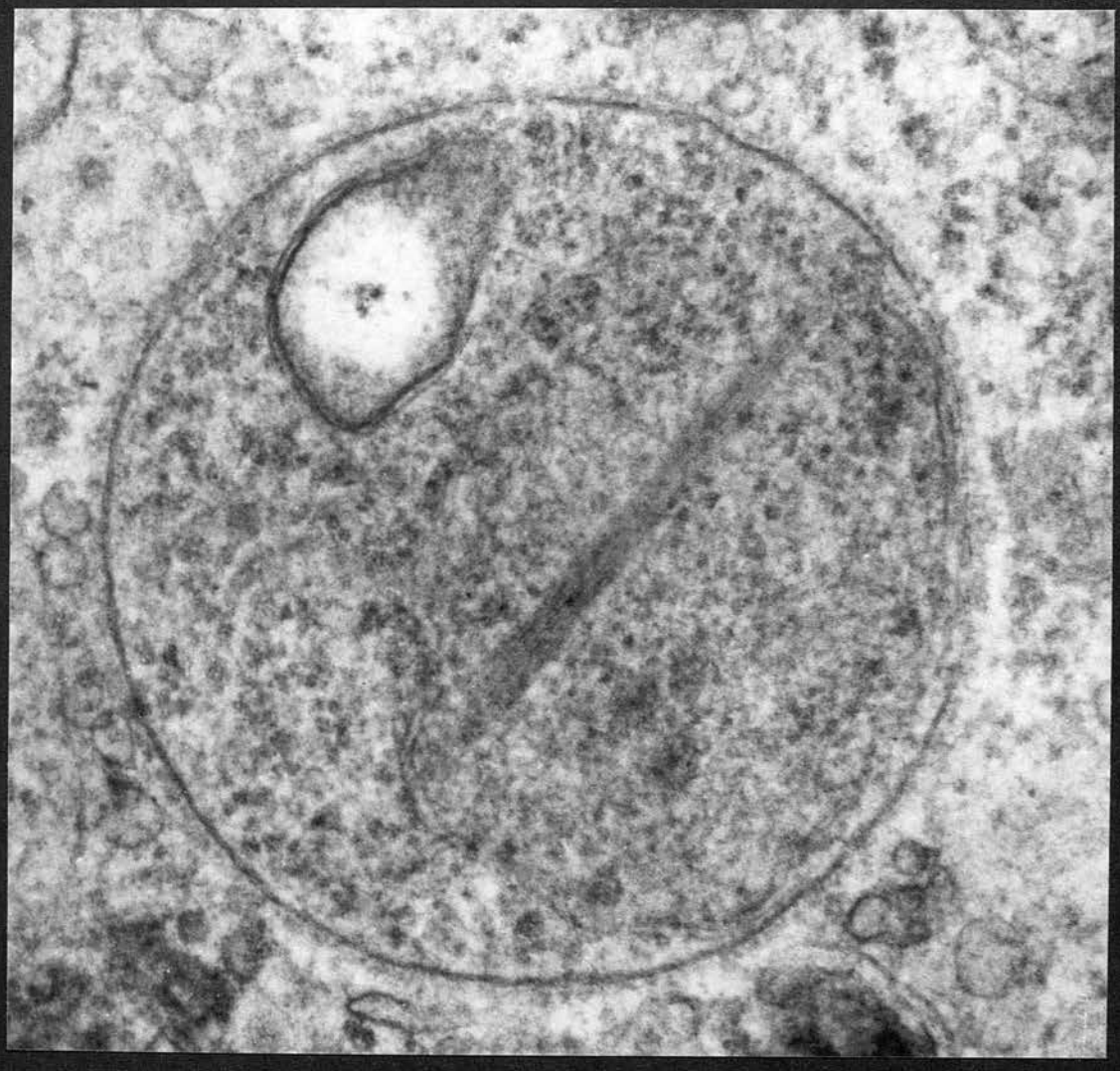


Figure 23 A binucleate schizont of T. annulata formed as a result of the acentric, closed mitosis with intranuclear spindle microtubule formation described in Figure 22.
Magnification, x 66,267

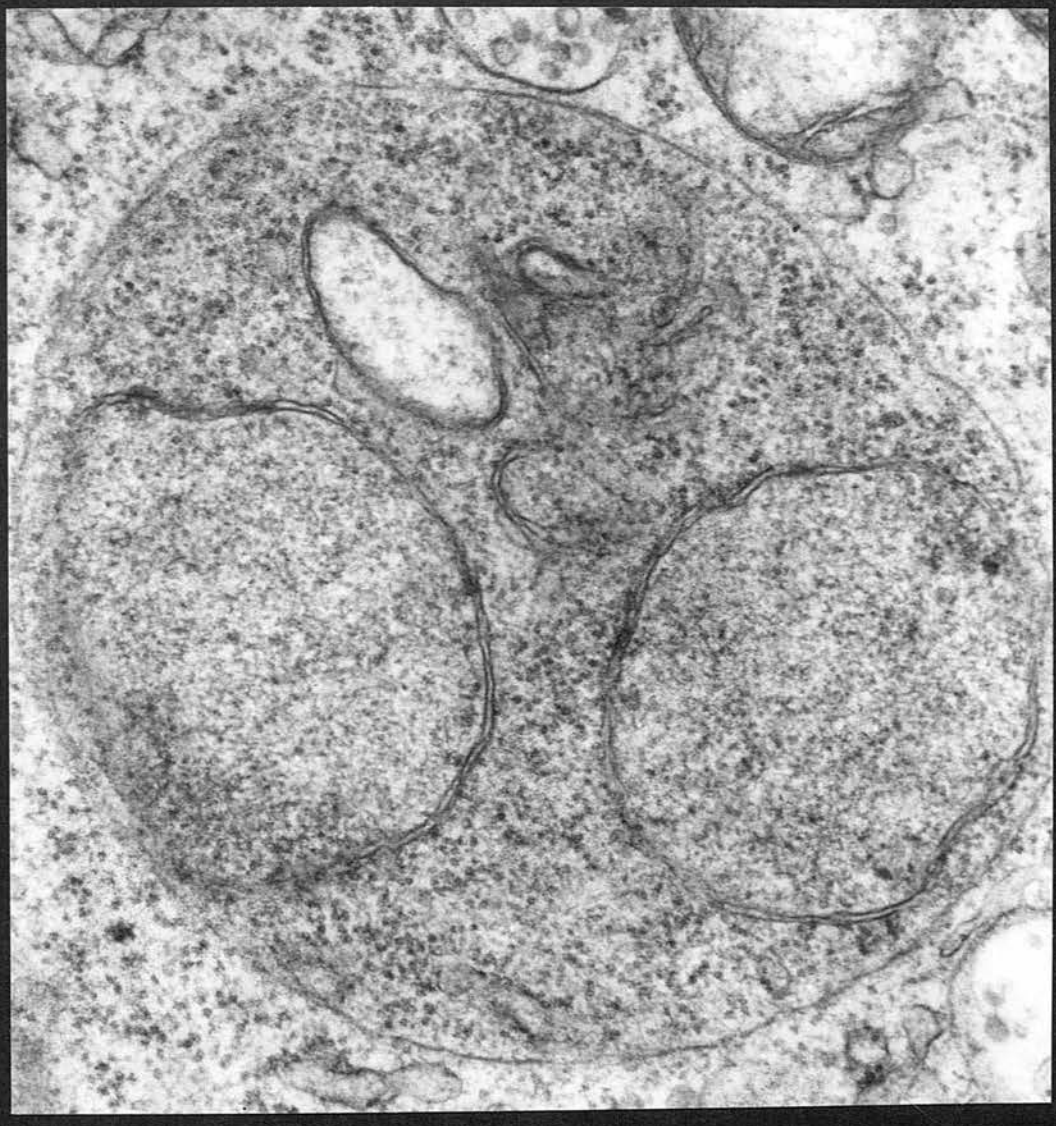


Figure 24 A T. annulata macroschizont, formed as a result of a closed mitotic replication of each nucleus of a binucleate schizont. Magnification, x 53,197

turn oxidises the polysaccharide to which it is bound. The overall reaction is an oxidation of the polysaccharide with an equivalent reduction of OsO_4 to lower insoluble oxidation products which are localised extracellularly. The exclusion of ruthenium red-OsO_4 reaction products delineates cell membranes and permits tracing tortuous plasmalemmal invaginations so that no intracellular staining is possible unless the continuity of the cell membrane is disrupted. The technique was employed in this investigation to ascertain whether the lymphocyte plasmalemma remains intact or is broken by T. annulata sporozoites during the interiorisation process.

4.4.2 Materials and methods

Cultures were established by incubating PBL prepared by defibrination with 8 μm filtrate of T. annulata (GUTS) (2 tick equivalents/ml) at 37°C. Samples were obtained at 15, 30 and 60 minutes of incubation. The ruthenium red technique was applied to these aliquots as described by Luft (1971). A stock solution of the dye was prepared as follows: (1) 50 mg of commercial ruthenium red (Johnson, Matthey and Company Inc., 608 Fifth Avenue, New York, USA) were weighed out and crushed in a mortar with a pestle, (2) a few drops of deionised, distilled water were added and crushing continued, (3) more distilled water was added and the suspension transferred to a graduated 15-ml centrifuge tube, with successive washing of the mortar until sufficient water (5 ml) was added to make 10 mg/ml final concentration, (4) the suspension was heated in a water bath at 60°C with frequent agitation for five minutes and centrifuged at 1,500 xg for ten minutes at 20°C (3,000 rpm, MSE

Chilspin), (5) the supernate was stored at 4°C as a stock solution.

The procedure for ruthenium red staining was carried out as described: (1) pellets of cultured cells were obtained as detailed in the chapter describing general materials and methods and fixed for one hour at 4°C in a freshly prepared mixture of 0.5 ml of 3.6% cacodylate-buffered glutaraldehyde, 0.5 ml of 0.2M cacodylate buffer, pH 7.4 and 0.5 ml of stock solution of ruthenium red, (2) they were rinsed in three changes of 0.2M cacodylate buffer over a ten minute period and fixed again at 20°C for three hours in a freshly prepared mixture of 0.5 ml of 5% OsO_4 in distilled water, 0.2M cacodylate buffer, pH 7.4 and 0.5 ml stock solution of ruthenium red solution, (3) this was followed by three changes of 0.2M cacodylate buffer, dehydration in graded series of ethanol and processing to araldite, embedding, cutting, staining and examination as described for routine electron microscopy.

4.4.3 Results

Complete extracellular localisation of reaction products of ruthenium red, with distinct delineation of lymphocyte plasmalemma was demonstrated by increased electron density of the membrane (Figure 25). Observations during the various stages of interiorisation of T. annulata sporozoites (Figures 26 and 27) showed that the cell membrane circumjacent to the invading parasite remained intact so that no intracellular ruthenium red-OsO_4 reaction products were observed. In cases where the sporozoites had been completely interiorised before staining (Figure 28), no ruthenium red was demonstrated in the host plasma membrane investing the parasite.

Figure 25 A non-infected bovine lymphocyte fixed and post fixed in glutaraldehyde-ruthenium red and OsO_4-ruthenium red mixtures respectively and processed to araldite by routine electron microscopic techniques. Complete localisation of reaction products of ruthenium red, with distinct delineation of lymphocyte plasmalemma is demonstrated by increased electron density of the membrane.
Magnification, x 23,571

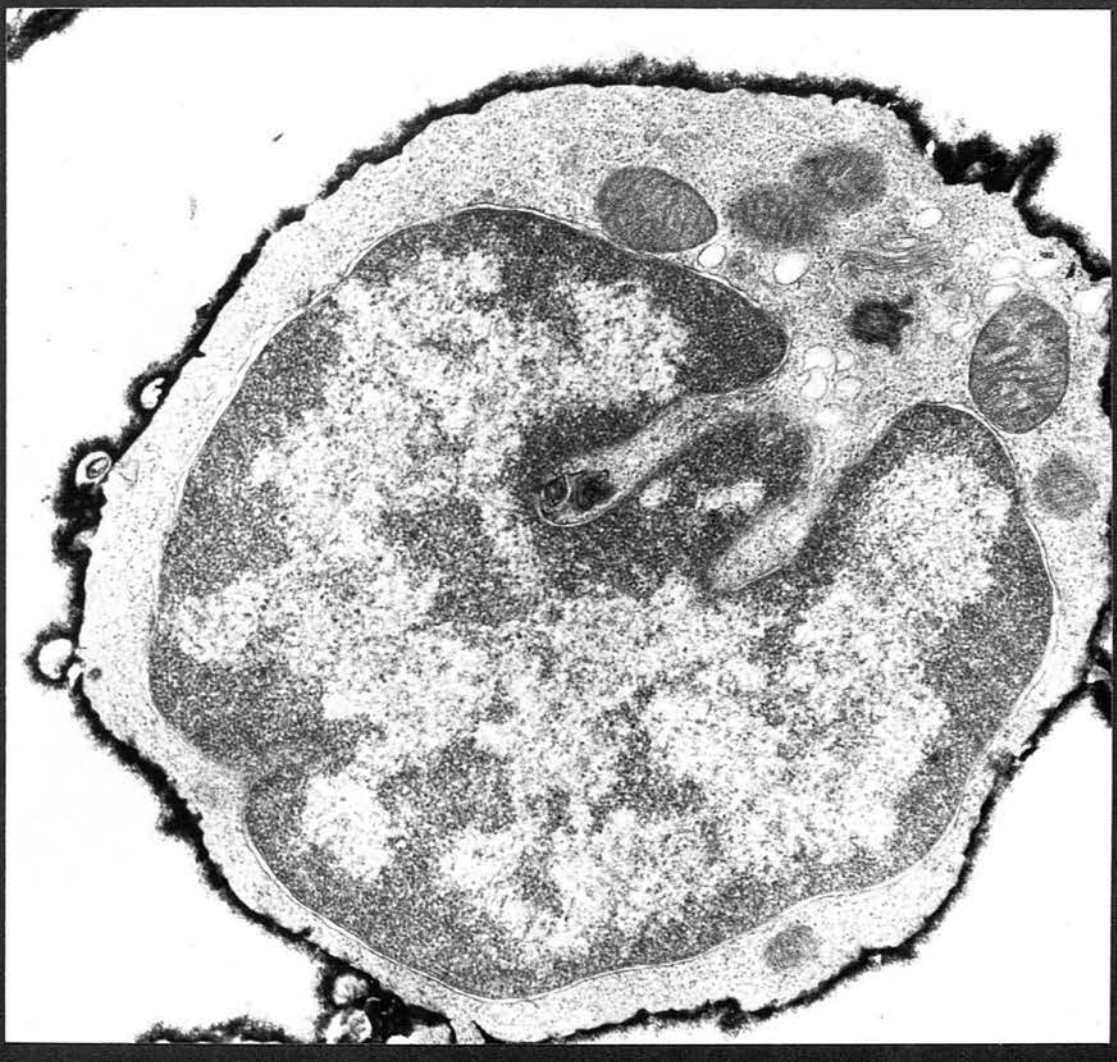


Figure 26 A *T. annulata* sporozoite in the process of interiorisation and stained with ruthenium red is illustrated. The target lymphocyte plasmalemma is deeply invaginated so that its ends are close to fusing around the invading parasite. The lymphocyte membrane circumjacent to the sporozoite remains intact throughout the process as demonstrated by lack of intracellular ruthenium red staining. Magnification, x 179,667

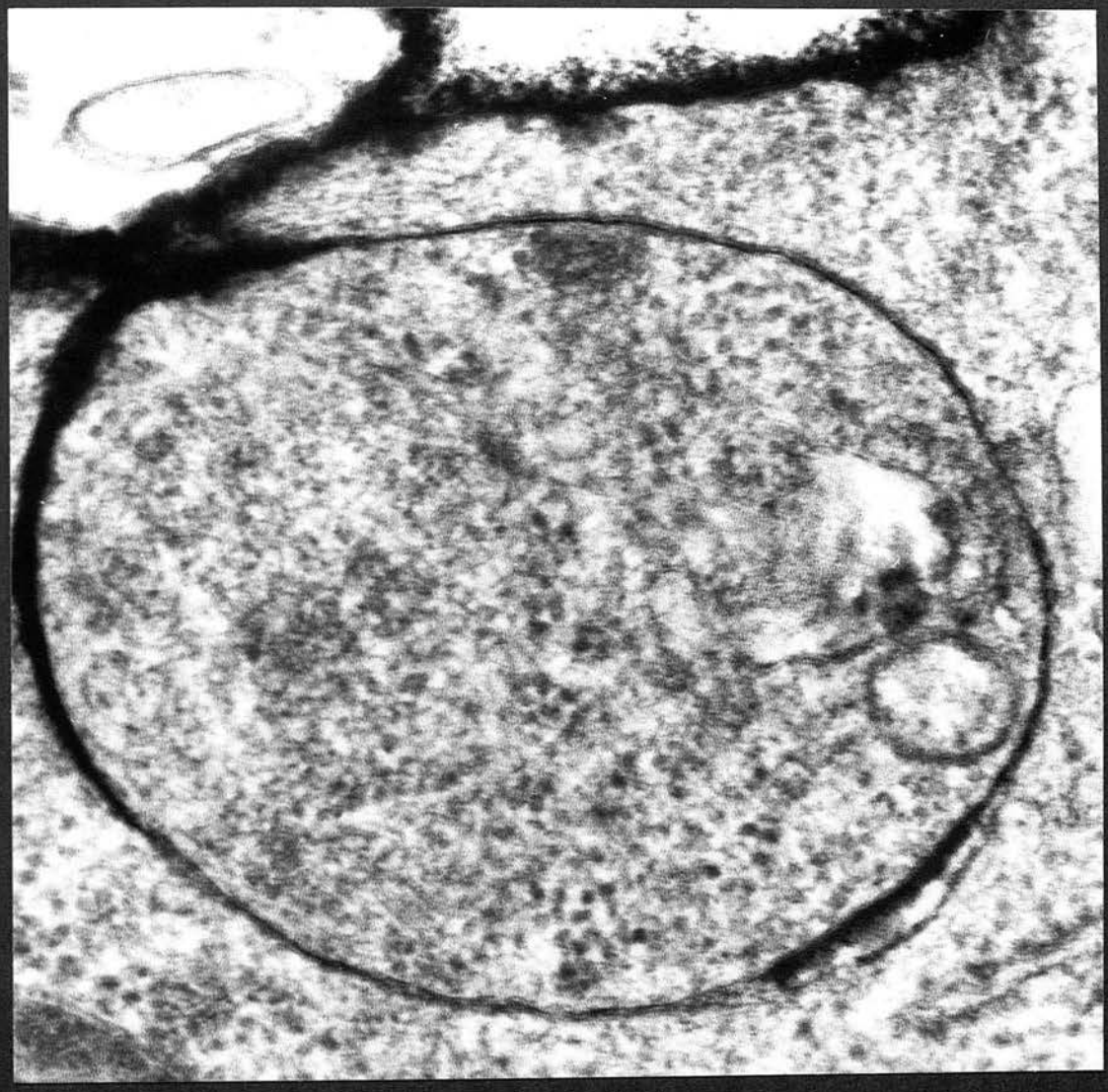


Figure 27 A T. annulata sporozoite processed with ruthenium red-fixative mixtures in the course of interiorisation is almost completely intracellular. The ends of the invaginating target lymphocyte membrane are touching but are not yet fused. The investing host cell membrane as well as parasite pellicle are delineated by electron-dense ruthenium red-OsO_4 reaction products. No reaction product deposits are intracellular, confirming that T. annulata sporozoites do not disrupt target cell membrane during the entry process. Magnification, x 128,333

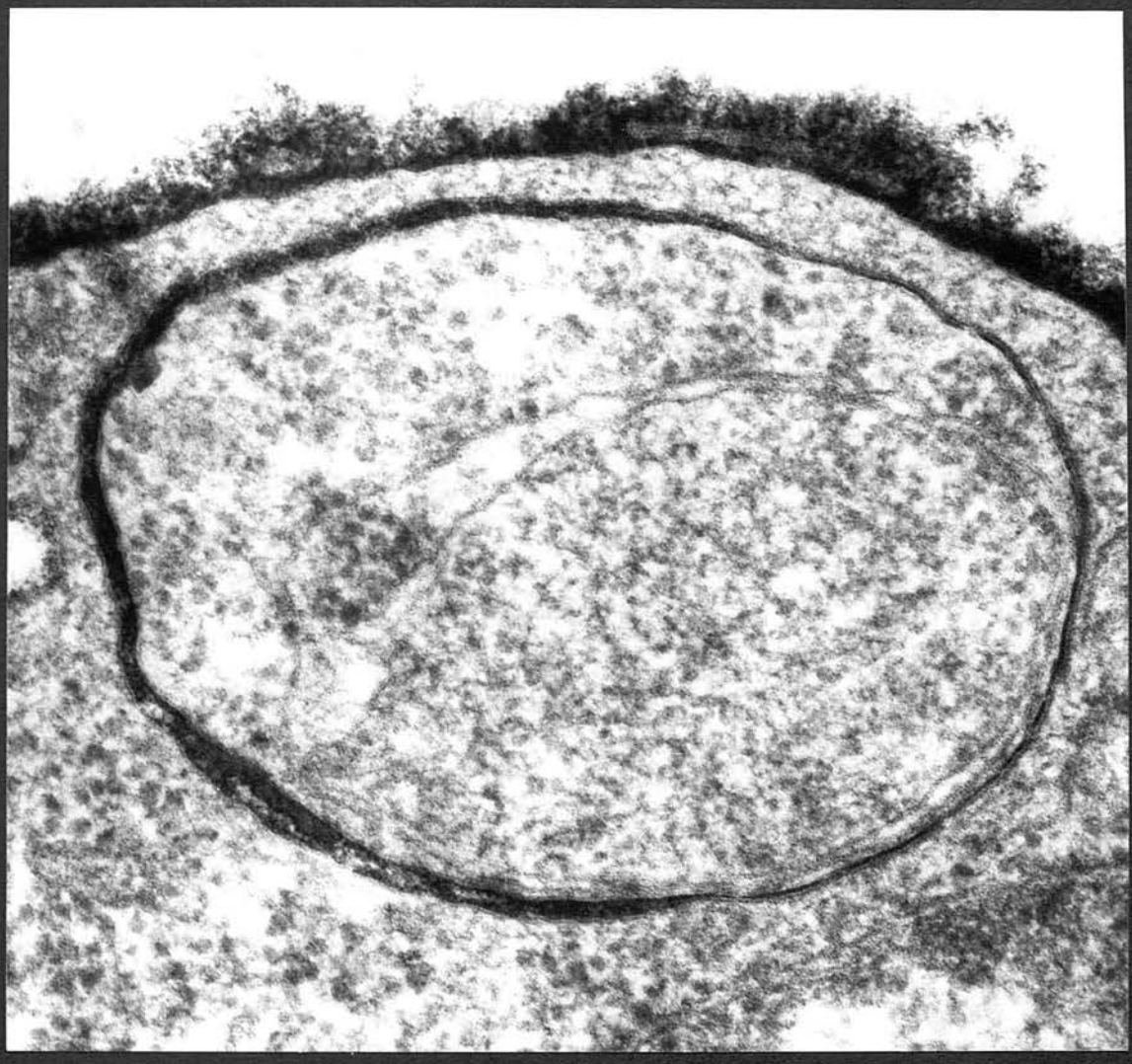


Figure 28 An electron micrograph showing intracellular and attached T. annulata sporozoites processed with ruthenium red-fixative mixtures. The intracellular parasite interiorised prior to ruthenium red treatment hence no reaction products are demonstrated either on its pellicle or on the circumjacent host membrane. Magnification, x 62,808

4.5 Effect of heat inactivation of T. annulata sporozoites on their ability to invade PBL

4.5.1 Introduction

Lymphocytes are generally not regarded as phagocytic. In this thesis, observations made in the preceding sections (Figures 15, 26 and 27) depicted T. annulata sporozoite pellicle and the susceptible bovine lymphocyte plasmalemma in close contact and no pseudopodia were observed during invasion and interiorisation of the parasite. Whether the entry of T. annulata sporozoites into bovine lymphocytes was solely parasite-effected or due to passive phagocytosis was not resolved. The purpose of this study was to resolve the issue by determining the ability of heat-inactivated T. annulata sporozoites to infect PBL in vitro.

4.5.2 Materials and methods

PBL separated from defibrinated blood, and GUTS filtrate of T. annulata, used at 2 tick equivalents/ml, were obtained as described under general materials and methods. 2.5 ml GUTS in a sterile, 15-ml conical centrifuge tube were placed in a thermostatically controlled, shaking water bath preset and stabilised at 60°C, and incubated for 45 minutes in order to inactivate T. annulata sporozoites. The inactivated sporozoites were allowed to cool to room temperature and distributed in 8, 15-ml centrifuge tubes each containing 0.25 ml. Another set of eight tubes was set up each containing 0.25 ml of fresh viable GUTS material. This latter set served as the control. To each tube in both the control and the heat-inactivated set, 0.25 ml fresh PBL suspension was added, mixed by pipetting, gassed at 5% CO_2/95% air and incubated at 37°C

for one hour. At the end of the incubation period, each tube received 5 ml growth medium and was centrifuged at 400 xg for five minutes (1,800 rpm, MSE Chilspin at 20°C). The pellets were resuspended in 0.5 ml complete RPMI 1640, cytocentrifuge smears prepared and stained with Giemsa stain. The number of interiorised sporozoites was determined out of 1,000 lymphoid cells in each of the eight replicate slides in both the control set and the set in which heat-inactivated sporozoites were used to infect PBL.

4.5.3 Results

The mean number of T. annulata sporozoites interiorised per 1,000 lymphocytes counted is presented in Table 2. Heat-inactivation of the sporozoites resulted into an almost total lack of invasion of fresh, normal PBL. The morphology of the sporozoites was apparently well preserved after heat treatment.

Table 2 Effect of heat-inactivation of T. annulata sporozoites on the number invading PBL per 1,000 lymphocytes after one hour

Category of sporozoites n = 8	Means and standard deviations
Viable sporozoites (control)	337 ± 63.10
Heat-inactivated sporozoites	0.8 ± 0.89

4.6 Effect of incubation temperature on the ability of T. annulata sporozoites to invade PBL

4.6.1 Introduction

The observation made above that heat inactivation of sporozoites almost completely precluded their interiorisation (Table 2) implied that the invasion of PBL is actively accomplished by T. annulata sporozoites. Active processes require active metabolism and are highly temperature-dependent (Silverstein, Steinman and Cohn, 1977). This experiment was consequently designed to investigate the influence of incubation temperature on the ability of T. annulata sporozoites to invade bovine peripheral blood lymphocytes.

4.6.2 Materials and methods

PBL, prepared from defibrinated blood, and GUTS of T. annulata sporozoites, used at 2 tick equivalents/ml, were obtained as described under general materials and methods. Three sets of cultures were established, each in replicates of eight. In one set, both sporozoites and lymphocytes were, initially, separately incubated at 0°C for one hour in melting ice then mixed and incubated at 0°C for an additional one hour. In the second set, sporozoites and PBL were kept separately at 37°C for one hour, rapidly mixed and incubated at 37°C for another hour. In the third, control set, sporozoites and lymphocytes were mixed at room temperature (20°C) and incubated at 37°C for one hour. In every case 0.25 ml PBL and 0.25 ml GUTS were mixed in a 15 ml, sterile centrifuge tube. At the end of the incubation period, cultures were centrifuged at 400 xg for five minutes (1,800 rpm, MSE Chilspin at 5°C), the supernate of sporozoites from each tube pipetted into fresh, sterile tubes and

kept while pellets were resuspended in 0.5 ml growth medium and cytocentrifuge smears made immediately for staining with Giemsa stain and quantitation of interiorised sporozoites. Four tubes with supernatant sporozoites from each of the three sets above were selected at random and used to establish cultures. To each of the randomly selected tubes with sporozoites, 0.5 ml fresh PBL were added, the mixture pipetted up and down several times and transferred to cluster plate wells in which feeder layers of fibroblastic cells of the Bison bison lung line, IMR31 had been established 24 hours previously. The plates were placed in a humidified plastic box, gassed at 5% CO_2/95% air and incubated at 37°C. The cultures were maintained as described under general materials and methods, and sampled for cytocentrifuge smears on Days 1, 3 and 4 for the determination of the number of infected cells. While the number of interiorised sporozoites was quantified out of 1,000 lymphocytes, the number of infected cells was determined per 200 cells.

4.6.3 Results

The mean numbers of sporozoites interiorised per 1,000 lymphocytes for cultures incubated at 0°C or 37°C as well as for the controls are presented in Table 3. One way analysis of variance revealed that differences between incubation temperatures were highly significant ($F = 292.00$; $P < 0.01$). Further analysis by Duncan's multiple range test revealed no significant difference between the cultures established from sporozoites and lymphocytes initially kept at 37°C, mixed and incubated at 37°C, and the controls in which sporozoites and lymphocytes were mixed at 20°C and immediately incubated at 37°C.

Table 3 Effect of temperature on the number of T. annulata sporozoites invading PBL per 1,000 lymphocytes at one hour

Incubation temperature n = 8	Means and standard deviations
0°C	14.5 ± 3.74
37°C	525.9 ± 41.79
Control	525.5 ± 73.47

A highly significantly smaller mean number of T. annulata sporozoites ($P < 0.01$) entered lymphocytes in cultures established by mixing and incubating sporozoites and lymphocytes at 0°C as compared to either the control or the group in which both sporozoites and lymphocytes were separately incubated at 37°C for one hour, mixed and incubated for an additional hour at 37°C. The mean numbers of infected lymphocytes determined per 200 cells on Days 1, 3 and 4 on cultures established using T. annulata sporozoite supernates derived from the three categories above (i.e. 0°C, 37°C and the control) are presented in Table 4. Two way analysis of variance revealed that no significant differences in the number of infected cells existed between the three categories.

Table 4 Virulence of T. annulata sporozoite supernates from 0°C, 37°C and control categories: mean number of infected PBL/200 cells

Origin of T. annulata sporozoite supernate n = 4	Days after establishing cultures		
	1	3	4
0°C	48.5 ± 5.82	47.8 ± 9.66	60.2 ± 13.04
37°C	47.5 ± 7.99	48.6 ± 9.60	57.4 ± 8.36
Control	44.6 ± 9.37	50.6 ± 9.57	59.4 ± 3.01

4.7 Influence of enzymatic treatment of PBL with trypsin on the invasive activity of T. annulata sporozoites

4.7.1 Introduction

The factors involved in the susceptibility of bovine lymphoid cells to infection by Theileria sporozoites are not known. It was demonstrated earlier in this chapter that sporozoites of T. annulata consistently attached to, and invaded, bovine peripheral blood lymphocytes by their basal end (Figures 14 and 15). The consistent base-first orientation was construed to implicate the existence of specific receptors on the plasma membrane of target bovine lymphocytes and reciprocal recognition sites on the basal aspect of the sporozoites. The availability of such surface determinants in the lymphocyte plasmalemma would influence its susceptibility to infection by T. annulata sporozoites. The elimination of the receptors from the lymphocyte surface should therefore abrogate recognition, attachment and invasion of the cells by T. annulata sporozoites. In this study, the effect of a mild tryptic proteolysis of the bovine lymphocyte plasmalemma would have on the invasive activity of T. annulata sporozoites was investigated.

4.7.2 Materials and methods

PBL, prepared from defibrinated blood, and GUTS of T. annulata sporozoites, used at 2 tick equivalents/ml, were obtained as described under general materials and methods. Trypsin from bovine pancreas Type III (CalBiochem., La Jolla, USA) was constituted in MEM with Earle's salts, pH 7.4, containing Ca^{++} as recommended by Gorini and Felix (1953). Ca^{++} inhibit autolysis of the enzyme which tends to concur with hydrolysis of a substrate thus permitting a

larger amount of trypsin to act on a substrate. The enzyme was constituted at double (e.g. 0.2, 2, 20, 200, 2,000 and 10,000 µg/ml) the desired final concentrations. Soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Missouri) was used at equimolar concentrations to trypsin. Four replicate cultures were set up in cluster plates for each trypsin concentration as well as the control. Each well received 0.25 ml PBL in MEM with Earle's salts, pH 7.4, containing 20% foetal calf serum, 100 iu/ml benzylpenicillin, 100 µg/ml streptomycin sulphate and 2 mM/ml L-glutamine, and 0.25 ml of the appropriate concentration of trypsin. Replicate wells for the controls only received 0.25 ml PBL. The plate was placed in a humidified plastic box, gassed with 5% CO_2/95% air and incubated at 37°C for 30 minutes. At the end of incubation period, each well containing trypsin-treated PBL received 0.5 ml soybean trypsin inhibitor solution in order to stop the proteolytic reaction due to trypsin. Cells were then inoculated with T. annulata sporozoites. While each well with trypsin-treated PBL received 1 ml GUTS, the control wells were inoculated with 0.25 ml of sporozoite suspension. The plate with cultures was again returned in a humidified plastic box, gassed as described above and incubated for one hour at 37°C. An additional set of control cultures was set up. 0.25 ml PBL suspension was mixed with soybean trypsin inhibitor, incubated for 30 minutes and then inoculated with 0.5 ml T. annulata sporozoite suspension and incubated at 37°C for one hour. At the end of the incubation, 50-µl aliquots of the cultures were centrifuged at 700 rpm for ten minutes onto microscope slides, air-dried, fixed in technical methanol and stained with Giemsa stain. The number of interiorised T. annulata

sporozoites was determined per 1,000 lymphoid cells counted for each replicate smear in each trypsin concentration and the two control groups.

4.7.3 Results

The means and standard deviations of the numbers of T. annulata sporozoites interiorised per 1,000 lymphocytes in cultures established using lymphocytes treated with various doses of trypsin, and in the controls are shown in Table 5. Increasing doses of trypsin significantly inhibited numbers of T. annulata sporozoites invading bovine peripheral blood lymphocytes. The relationship of numbers of interiorised sporozoites to dose of trypsin was curvilinear even when dose was transformed into logs ($F^{4}_{18} = 4.14$; $P < 0.05$). A linear relationship was, however, depicted (Figure 29) using a log-log transformation ($F^{4}_{18} = 1.64$; $P > 0.05$). Even the lowest dose of trypsin significantly reduced the numbers of T. annulata sporozoites entering lymphocytes as compared to the controls ($F^{2}_{9} = 50.06$; $P < 0.01$). The difference between controls was not significant as judged by Duncan's multiple range test.

Figure 29 The relationship of the number of interiorized T. annulata sporozoites to the dose of trypsin. A log-log transformation depicts a linear relationship.

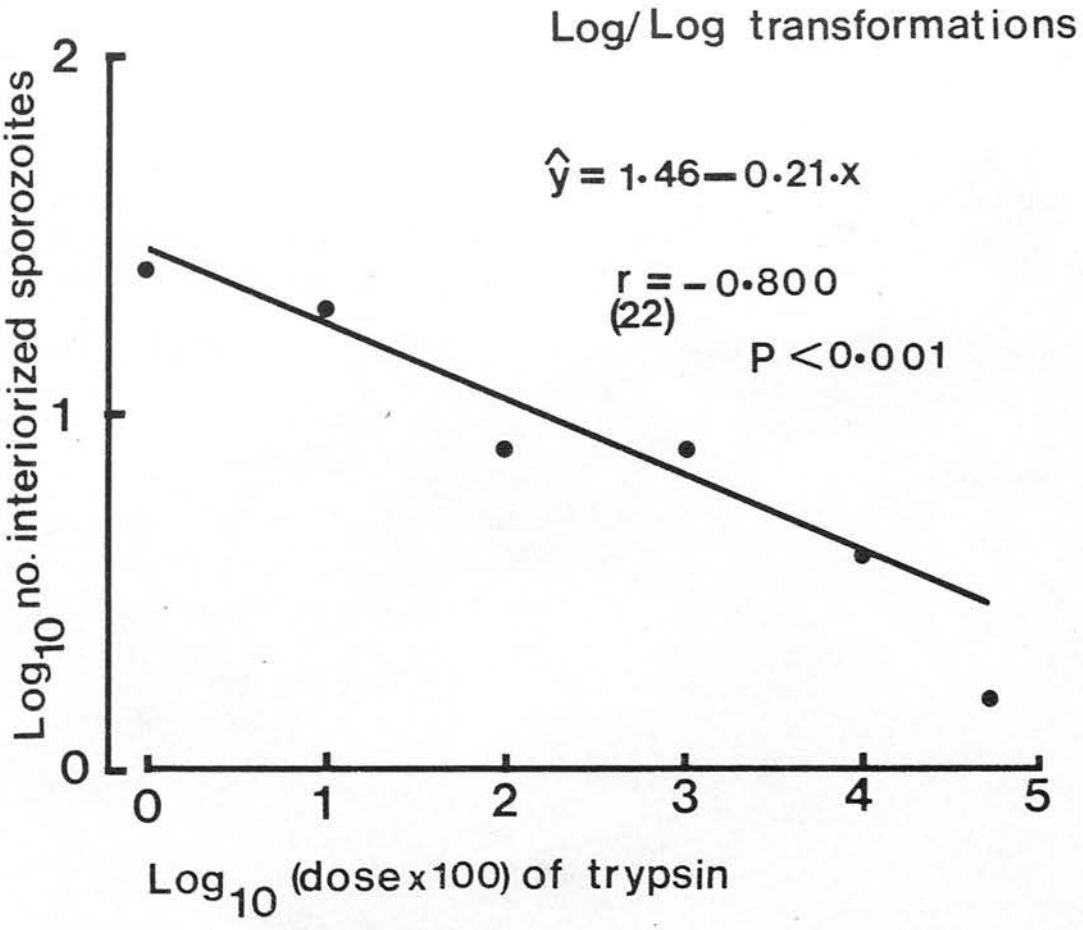

Log/Log transformations
$\hat{y} = 1.46 - 0.21.x$
r = −0·800
(22)
P < 0·001
Log_{10} no. interiorized sporozoites
2
1
0
0
1
2
3
4
5
Log_{10} (dose x 100) of trypsin

Table 5 Effect of pretreating PBL with trypsin on the number of T. annulata sporozoites invading per 1,000 lymphocytes

Trypsin (μg/ml) n = 4	Means and standard deviations
0[1]	348.0 ± 62.40
0[2]	355.5 ± 67.00
0.1	27.3 ± 5.91
1.0	19.8 ± 4.99
10	7.5 ± 3.42
100	7.3 ± 2.22
1000	4.3 ± 1.26
5000	1.8 ± 1.71

[1] 1st control (PBL + T. annulata sporozoites)

[2] 2nd control (PBL + Soybean trypsin inhibitor + T. annulata sporozoites)

4.8 Encounter of, and interaction with, host lymphocyte lysosomal system by the developing T. annulata sporozoites

4.8.1 Introduction

It has been demonstrated cytochemically that acid phosphatase activity resides in about 80% of normal, "resting" T lymphocytes and 45% of B cells and is most frequently observed in secondary lysosomes of varying size and content (Poore, Barrett, Kadin and Bainton, 1981). Some lymphocytes contained reaction product for acid phosphatase within endoplasmic reticulum and the perinuclear cisterna indicating that they are actively synthesizing acid phosphatase. In phytohaemagglutinin-stimulated lymphocytes (Hirschhorn,

Hirschhorn and Weissman, 1967) biochemical assays have demonstrated a marked net increase in two lysosomal acid hydrolases: acid phosphatase and aryl sulfatase. Lysosomal structures containing these acid hydrolases normally fuse with, and discharge their contents into, phagosomes, thus producing digestive vacuoles or phagolysosomes within which degradation of ingested materials takes place. Earlier in this chapter (Figure 20) it was demonstrated that the developing T. annulata trophozoite provokes a massive lysosomal reaction within the host lymphocyte. The trophozoite, however, circumvented the observed lysosomal armamentarium and developed into the multinucleate schizont stage (Figures 23 and 24). How the intralymphocytic stages of T. annulata evade the cytocidal activity of host cell lysosomes has not been defined. The aim of this study is to illustrate, by acid phosphatase ultracytochemistry, how the developing parasite interacts with the host lysosomal system.

4.8.2 Materials and methods

PBL, separated from defibrinated blood, and GUTS of T. annulata sporozoites, used at 2 tick equivalents/ml, were obtained as detailed under general materials and methods. Fourteen cultures, seven controls and seven tests, were established in a multi-well cluster plate by mixing 0.25 ml (2×10^6 cells) PBL suspension with 0.25 ml T. annulata sporozoite suspension. The plate with cultures was then placed in a humidified plastic box, gassed with 5% CO_2/95% air and incubated at 37°C. Sampling for acid phosphatase ultracytochemistry was carried out at 1, 3, 6, 18, 24, 48 and 72 hours. Fresh non-infected PBL were processed immediately and examined for

the presence of acid hydrolase enzymes. A modification of the technique recommended by Barka and Anderson (1962) was used for the cytochemical demonstration of lysosomal activity within infected and non-infected cells. After appropriate incubation periods, two cultures were transferred into plastic, disposable universal bottles, centrifuged at 275 xg for ten minutes (1,450 rpm, MSE Minor at 20°C) and the supernate discarded. The pellets were resuspended in serum-free Eagle's MEM with Earle's salts and centrifuged again at 275 xg for five minutes at 20°C (1,450 rpm, MSE Minor). The resultant pellets were fixed in 1.5% glutaraldehyde in 0.1M sodium cacodylate-HCl buffer, pH 7.4, containing 1% sucrose at 4°C for two hours. The pellets were washed several times in 0.1M sodium cacodylate-HCl buffer, pH 7.4, containing 1% sucrose at 4°C. They were then washed for 20 minutes in sucrose-Tris/maleate buffer composed of 12.5 ml 0.2M Tris/maleate buffer, pH 5.2, 37.5 ml deionised distilled water and 4.2 g sucrose. While one of the pair of the washed pellets was treated as a test sample, the remainder was processed as a control and incubated at 37°C for one hour in an incubation medium lacking the substrate, sodium-β-glycerophosphate. The test pellet was incubated for one hour at 37°C in a complete incubation medium comprising (a) 10 ml 0.2M Tris/maleate buffer, pH 5.2, (b) 4 ml 0.1M sodium-β-glycerophosphate, (c) 6 ml 0.02M lead nitrate, (d) 30 ml distilled water and (e) 4.2 g sucrose. After incubation, both control and test pellets were washed over a period of one hour in three changes of sucrose-Tris/maleate buffer, post-fixed in a 2:1 mixture (v/v) of 1% OsO_4: 2.5% glutaraldehyde in 0.1M sodium cacodylate-HCl buffer, pH 7.4 for 1½ hours. The pellets were then washed in three changes

of 0.1M cacodylate buffer, pH 7.4, and stained en bloc with 0.5% buffered uranyl acetate (Watson, 1958) containing 4% sucrose for one hour at 20°C. The samples were dehydrated in graded series of ethanol and embedded in araldite as detailed under general materials and methods. Sections for electron microscopic examination, cut with LKB glass knives on a Cambridge-Huxley ultramicrotome and mounted on 200 mesh copper grids, were stained briefly (1-2 minutes) in lead citrate (Reynolds, 1963). Double staining with uranyl acetate was avoided as it would increase the density and make it difficult to recognise the reaction product.

4.8.3 Results

The acid phosphatase reaction product, lead phosphate, was demonstrated in the majority of non-infected bovine peripheral blood lymphocytes (Figures 30 and 31). Although lead phosphate deposits were most frequently demonstrated in secondary lysosomes, in some instances (Figure 30) they were found localised within profiles of rough endoplasmic reticulum and the Golgi complex. Samples incubated in the medium lacking sodium-β-glycerophosphate did not manifest any lead phosphate precipitates. In almost every instance the trophozoite stage of T. annulata provoked intense lysosomal activity within the host lymphocyte. The activity was demonstrated within both the host cell and the parasites (Figures 32 and 33). In the parasite, the electron-dense lead phosphate deposits were often seen associated with the nuclear membrane and rough endoplasmic reticulum (Figure 33). Secondary lysosomal bodies failed to fuse with morphologically viable trophozoites (Figures 32, 33 and 34). Dead trophozoites, however, readily fused with lysosomes (Figure 35) so that acid phosphatase activity was diffusely disseminated within the degenerating parasite.

Figure 30 A non-infected bovine peripheral blood lymphocyte processed to demonstrate acid phosphatase activity. Electron-dense lead phosphate deposits showing acid phosphatase activity are localised in secondary lysosomes, occasional strands of endoplasmic reticulum and what appears to be a saccule of the Golgi complex.
Magnification, x 39,929

Lead phosphate deposits arrowed

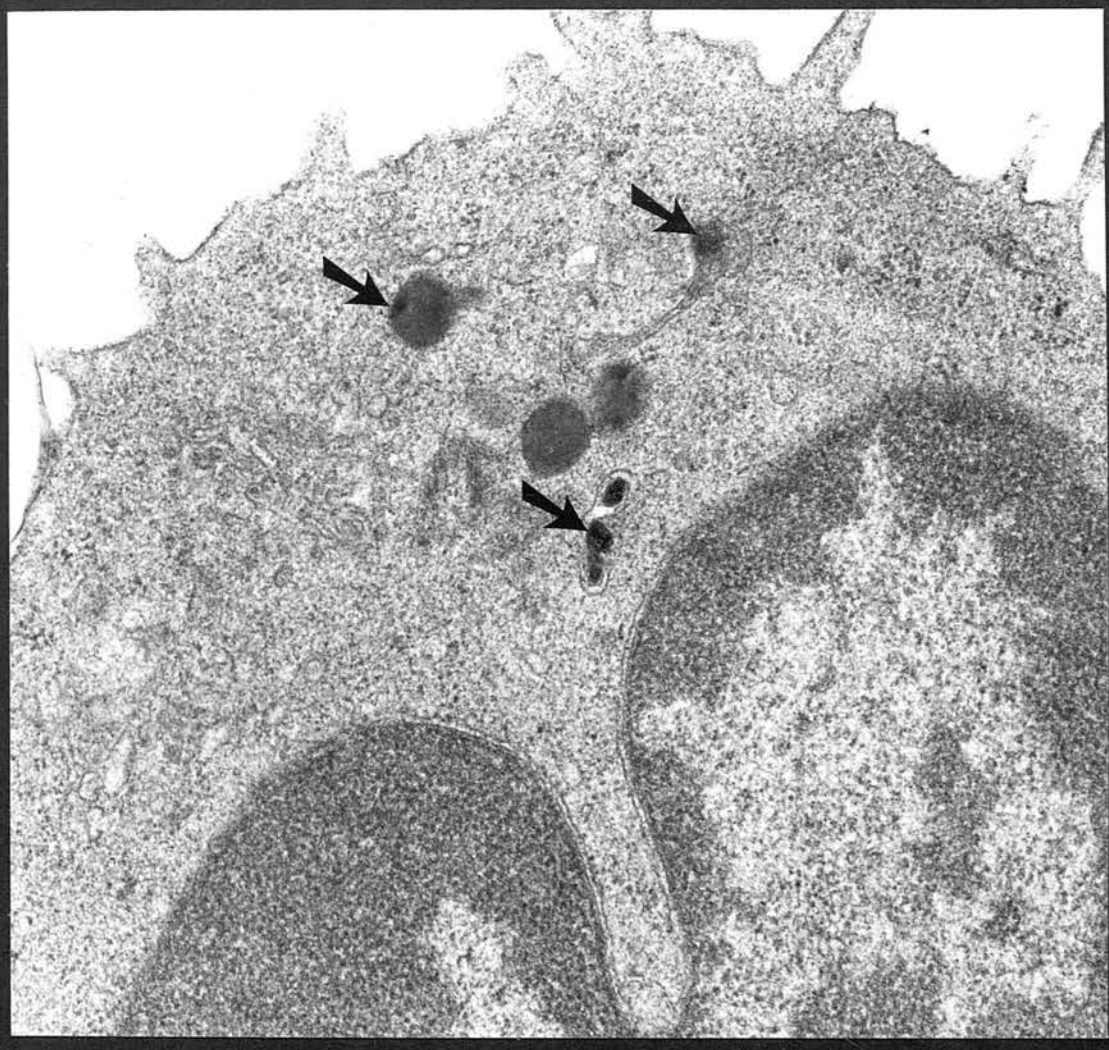


Figure 31 Large electron-dense deposits of lead phosphate demonstrating acid phosphatase activity within secondary lysosomes. Magnification, x 44,536

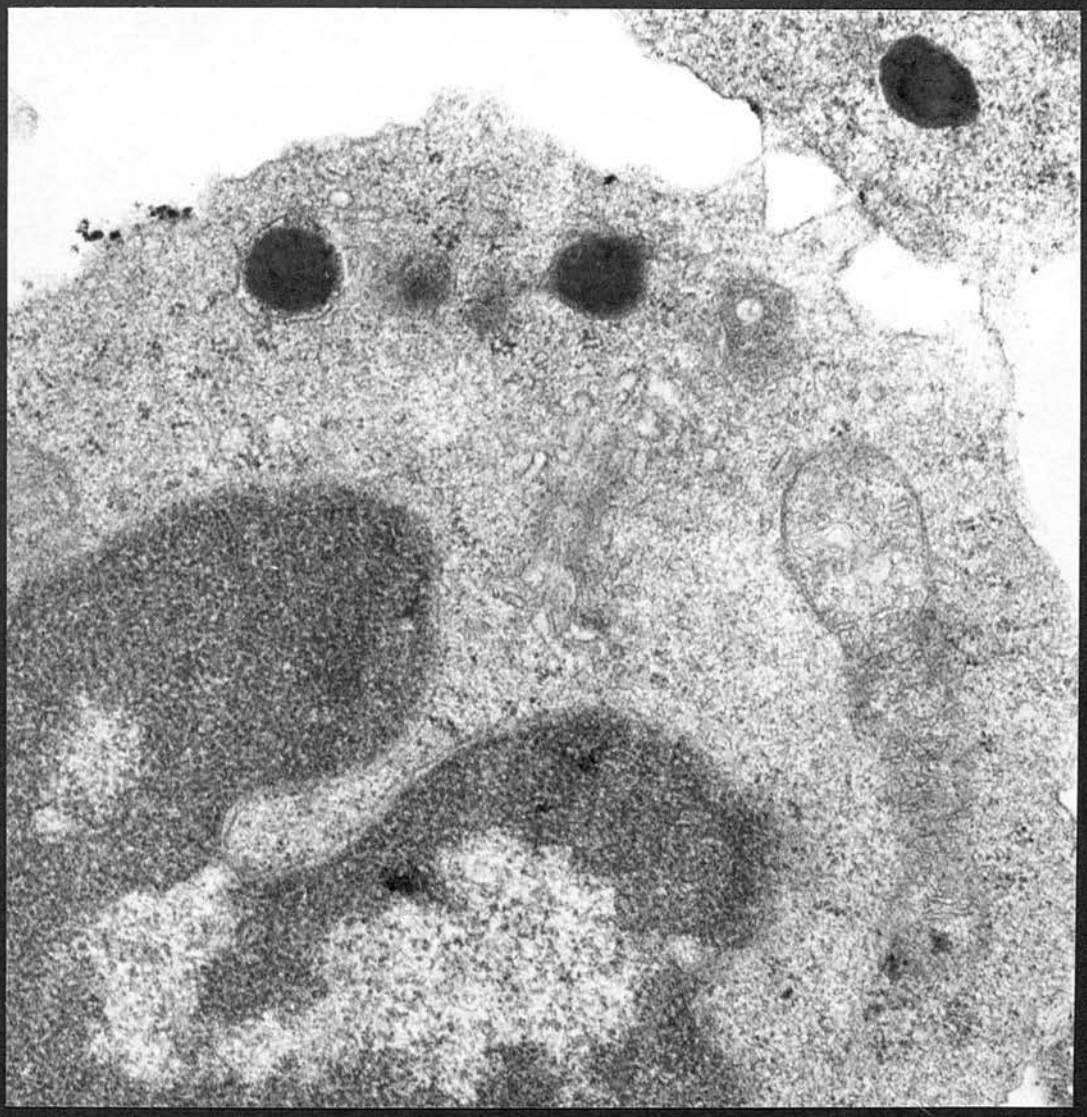


Figure 32 A T. annulata trophozoite within its host lymphocyte around 24 hours of incubation. Acid phosphatase activity is demonstrated both within the host cell as well as in the cytoplasm of the parasite. A section of a bundle of intranuclear microtubules is manifest in the parasite.
Magnification, x 92,885

Lead phosphate deposits arrowed

Figure 33 Electron-dense deposits of lead phosphate demonstrating acid phosphatase activity are illustrated associated, in T. annulata trophozoite at 18-24 hours, with the parasite nuclear membrane and rough endoplasmic reticulum. Host secondary lysosome, illustrated in close proximity to the parasite, has failed to fuse with it.
Magnification, x 125,000

Lead phosphate deposits arrowed

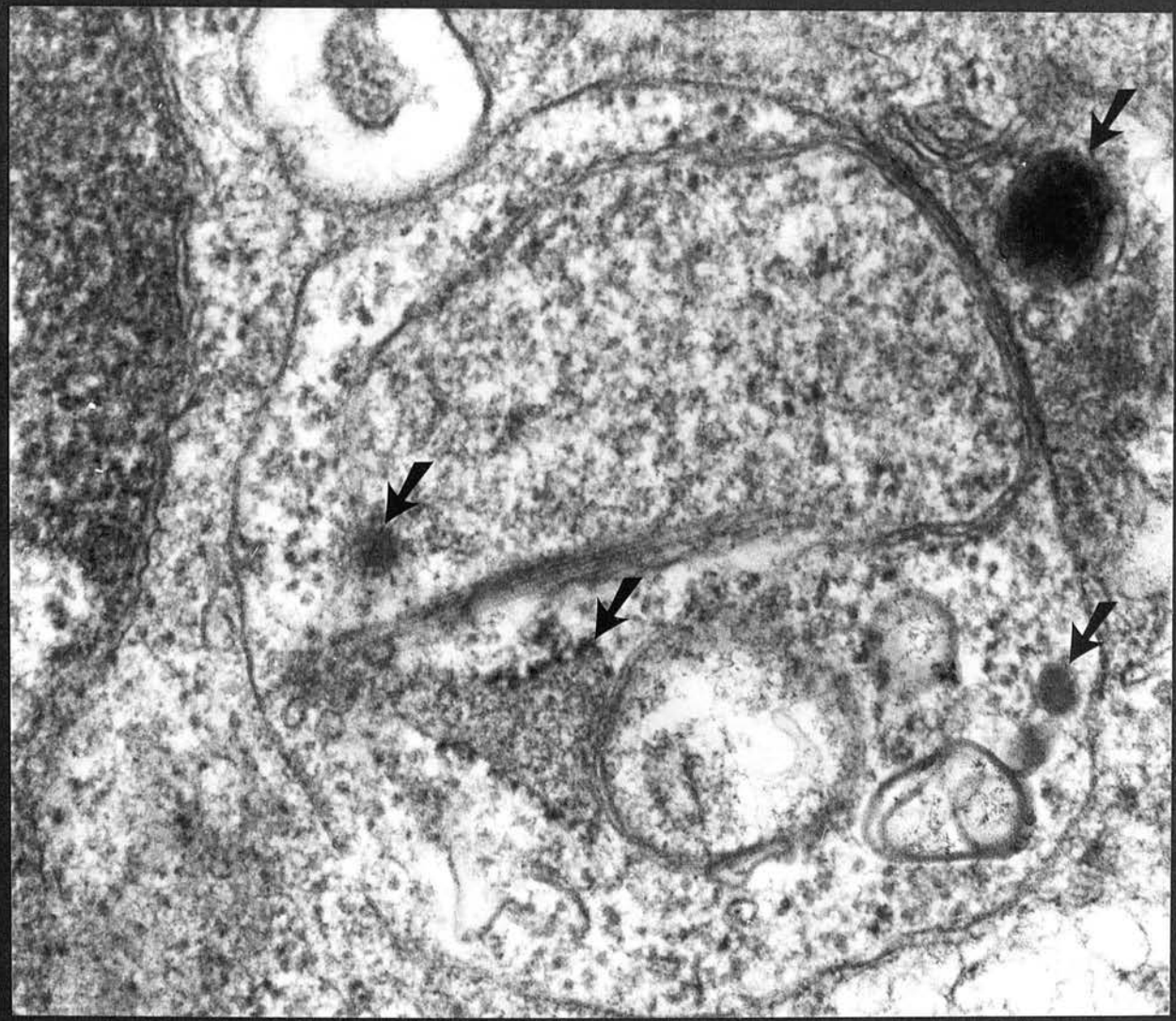
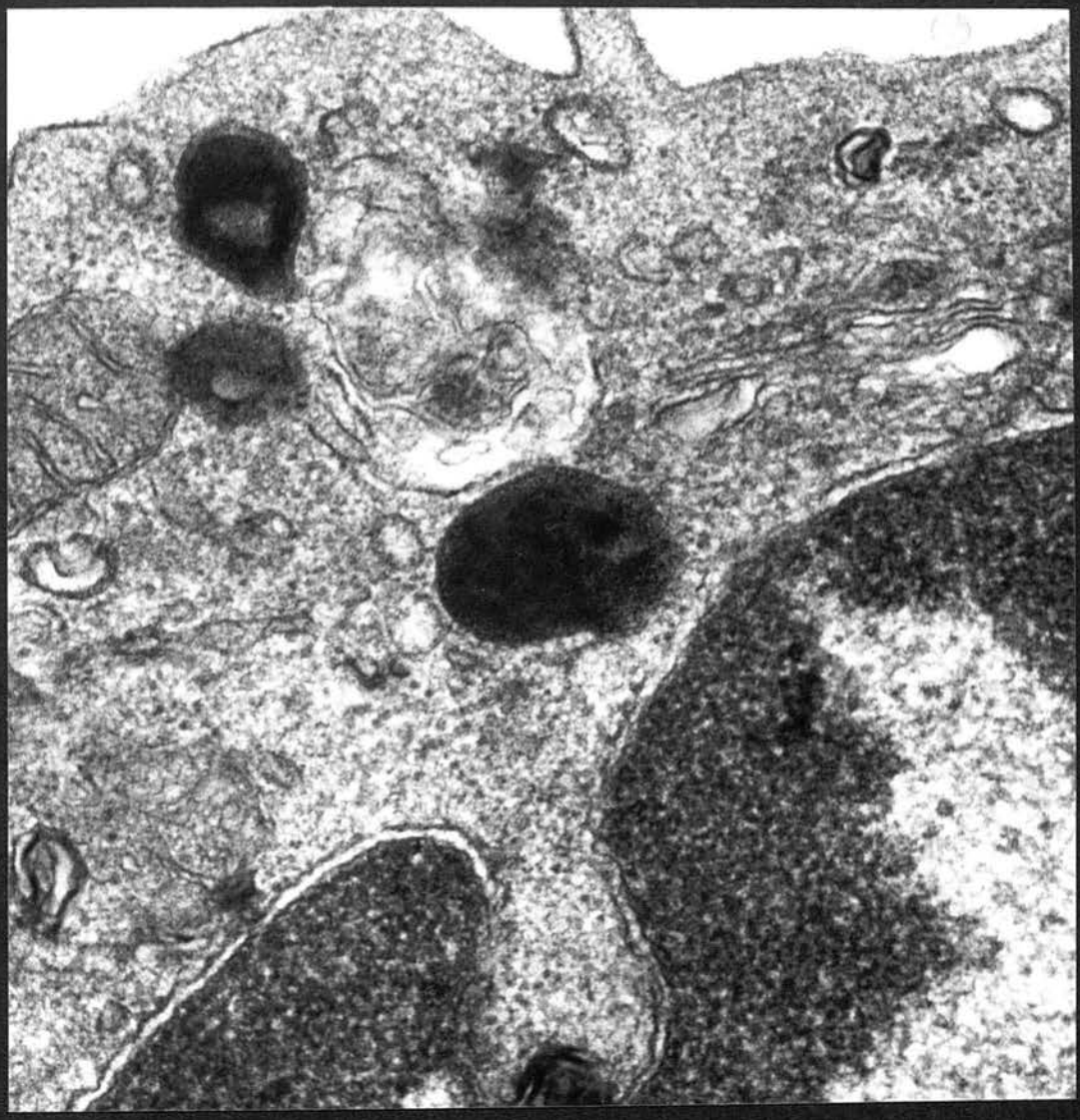


Figure 34 An electron micrograph of developing T. annulata trophozoites within the host cell cytoplasm at 18-24 hours of incubation. Host cell secondary lysosomes laden with lead phosphate deposits are shown in the vicinity of the parasites. No fusion takes place.
Magnification, x 59,038

Figure 35 A dead T. annulata trophozoite has fused with host lysosomes and lead phosphate deposits depicting acid phosphatase activity are disseminated within the degenerating parasite. Magnification, x 94,300



4.9 Discussion

The fine structural characteristics of T. annulata sporozoites have been defined in this study. It has been shown that this stage of the parasite is delimited by a thin, continuous, unit membrane, the pellicle, which is not only devoid of a surface coat and a cytostome, but also lacks additional supporting membranes and microtubules on its inner aspect. It has also been demonstrated that while the parasite possesses an apical complex comprising several rhoptries and a short, subpellicular membranous layer, the polar ring, it lacks the conoid (Gustafson, Agar and Cramer, 1954). While the mitochondria are tubular and acristate, the nucleus, which contains no nucleoli, is vesicular and non-chromocentric, manifesting no heterochromatic clumps. The cytoplasmic matrix of a T. annulata sporozoite, although devoid of endoplasmic reticulum and the Golgi complex, contains numerous ribosomes.

Ultrastructural criteria to be considered for the taxonomy of the Sporozoea were outlined by Vivier (1970), and included the fine structure of the dissemination forms such as sporozoites and merozoites, as well as some cytological characteristics of the vegetative stages. On the basis of these criteria the Theileriidae Du Toit, 1918 and the Babesiidae Poche, 1913 have recently been classified together (Levine et al., 1980) in the subclass Piroplasmia Levine, 1961 within the class Sporozoea Leuckart, 1879 and the phylum Apicomplexa Levine, 1970.

Sporozoites as well as merozoites of the genus Babesia Starcovici, 1893 manifest similar ultrastructural features (Friedhoff, 1974; Potgieter and Els, 1977 and Scholtyseck, 1979). These include a

pellicular complex consisting of an outer, continuous unit membrane and two thick, interrupted inner membranes; polar rings which may be found either anteriorly or both anteriorly and posteriorly; rhoptries and micronemes; mitochondria; a vesicular nucleus without heterochromatic clumps; a spherical body located between the anterior pole and the nucleus; and poorly defined but occasionally well developed endoplasmic reticulum. Although a conoid is normally not observed, Buttner (1968) not only reported its dubious occurrence in Babesia canis, but also demonstrated the presence of microtubules in the same parasite. Subpellicular microtubules have also been reported in Babesia major (Morzaria, Bland and Brocklesby, 1976) and Babesia bigemina (Potgeiter and Els, 1977a). Sporozoites of T. annulata differ structurally from the dissemination forms of Babesia species in that they lack the inner membrane complex, subpellicular microtubules which are, however, only observed in some Babesia species, spherical body, rough endoplasmic reticulum, a surface coat and, like almost all Babesia species, a conoid. T. annulata sporozoites, however, possess similar ultrastructural features as described in sporozoites of T. parva (Fawcett et al., 1982a) apart from the reported absence of a polar ring, and presence of an inconspicuous surface coat in the latter parasite. Fine structural observations of Schein et al. (1978) on the merozoites of T. annulata. T. lawrencei, T. mutans and T. parva revealed much greater similarity between the dissemination forms of Theileria and Babesia parasites. Even though the Theileria merozoites lacked endoplasmic reticulum, spherical body and a conoid, they, like their Babesia counterparts, possessed a unit membrane underlain by two, interrupted inner membranes, an anterior polar ring from which subpellicular microtubules

originated, 3-4 rhoptries with few micronemes, double-membraned mitochondria and, a vesicular nucleus with a homogenous karyoplasm.

It has been shown in this study that some T. annulata sporozoites seen in close proximity to bovine lymphocytes developed pseudopod extensions of their pellicle on the basal aspect subjacent to the nucleus (Figure 13). It has also been demonstrated that prior to interiorisation, T. annulata sporozoites must come in contact with the plasmalemma of the target bovine lymphocyte in a particular orientation. They consistently attached to the host plasma membrane by their basal end (Figures 14 and 28). The significance of the pseudopod development by the sporozoites is not clear. Pseudopods are believed to be ordinarily absent in the Sporozoea and, if present, are thought to be for feeding and not for locomotion (Honigberg et al., 1964; Levine et al., 1980).

In this study, however, it would appear to represent a locomotory organelle developed as a result of parasite-host recognition, and facilitating the advancement of the sporozoite for its eventual attachment to the target lymphocyte. The consistent base-first orientation during the attachment of T. annulata sporozoites to bovine lymphocytes implicates the presence of specific receptor sites on the plasmalemma of target host cell and reciprocal recognition sites on the basal aspect of the sporozoites. The availability of such surface determinants in the lymphocyte plasma membrane would influence its susceptiblity to infection while their distribution would determine the pattern of attachment of sporozoites to the cell surface. It has been observed in this study that only a proportion of lymphocytes possess suitable surface receptors since even after

18 hours of incubation only 14% were susceptible to infection by T. annulata sporozoites and two subpopulations of susceptible cells were observed: one in which sporozoites penetrated at one pole of the lymphocyte and the second group in which sporozoites attached and penetrated more evenly around the plasmalemma (Figure 12). It would, therefore, be reasonable to suggest that the susceptible bovine lymphocytes possessed reciprocal receptor sites to those on the basal aspect of T. annulata sporozoites, and that the two patterns of sporozoite attachment observed in this study (Figure 12) were related to the distribution of receptor sites on the host lymphocyte plasma membrane. The observation made in this investigation that pretreatment of bovine lymphocytes with trypsin resulted in a highly significant annulment of their recognition and invasion by T. annulata sporozoites (Table 5) strongly incriminates the involvement of specific surface proteins or glycoproteins as the receptors. The ability of haemosporozoans of the genera Plasmodium and Babesia to attach to, and subsequently penetrate, mammalian erythrocytes is also directly related to their ability to recognise specific determinants on the host cell membrane. Individual species of Plasmodium are highly host-specific (Garnham, 1966). The specificity of plasmodial-erythrocyte interactions has been shown to be related to specific components on the red cell membrane (McGhee, 1953; Butcher, Mitchell and Cohen, 1973; Miller, Dvorak, Shiroishi and Durocher, 1973). Whereas Plasmodium knowlesi parasites interact with Duffy blood groups (Miller, Mason, Dvorak, McGinniss and Rothman, 1975), Plasmodium falciparum merozoites appear to interact with a glycoprotein on the erythrocyte surface (Miller, Haynes, McAuliffe,

Shiroishi, Durocher and McGinniss, 1977). In Babesia rodhaini infection, receptors dependent on C3b component of the complement have been shown to be necessary for the penetration process (Chapman and Ward, 1977).

It has been demonstrated in this study that, following their attachment to target lymphocytes, T. annulata sporozoites achieve interiorisation by progressively invaginating the plasmalemma of the host cell (Figure 15). It has also been demonstrated by ruthenium red staining that during the interiorisation, the dye is completely excluded from the intracellular milieu, indicating that the host cell membrane remains intact throughout the process (Figures 26 and 27). While the observation made here, that heat-inactivation of T. annulata sporozoites abolished their interiorisation (Table 2), indicates that the entry process is parasite-effected, the highly significant reduction in the number of interiorised sporozoites by incubation at $0^{\circ}C$ (Table 3) suggests that the invasion of bovine lymphocytes of T. annulata is an active process dependent on active metabolism. The few sporozoites which entered lymphocytes in cultures incubated at $0^{\circ}C$ could have been activated by the warmth generated during centrifugation. That the incubation at $0^{\circ}C$ only blocked the interiorisation process but did not impair the virulence of T. annulata sporozoites is illustrated in Table 4 where supernatant sporozoites from cultures incubated at $0^{\circ}C$ were equally as infective as those obtained from the control and $37^{\circ}C$ categories.

The host cell membrane acquired by sporozoites during entry has been shown to fragment and disappear 30 minutes later when the invading parasites are already within the cytoplasm of the host

lymphocyte (Figure 16) so that the parasites are left in direct contact with the host cytosol. At the same time, it has been shown that the interiorised T. annulata sporozoites round up, lose their rhoptries and thus transform into trophozoites (Figure 16). The trophozoites not only commence feeding on chunks of host cell cytoplasm by phagotrophy through their cytostomes (Figure 19) but also engage in active synthesis of endogenous proteins for growth, as well as secretory and export proteins, as demonstrated by the development of polyribosomes and elaborate rough endoplasmic reticulum (Figures 17 and 18). How the parasite moves within the host cytoplasm to occupy the much preferred juxtanuclear position is not clear. The observation made in this study that the trophozoite develops a pseudopod pellicular extension (Figure 21) suggests this as a means of movement.

It has been shown in this study (Figure 22) that nuclear division in T. annulata is characterised by (a) the development of intranuclear, pole-to-pole spindle microtubules anchored at both ends in spindle pole bodies which are closely associated with parasite nuclear membranes, (b) lack of chromosomal uncoiling and condensation, and (c) lack of fragmentation of the nuclear envelope. Since the nuclear envelope remains intact and the spindle microtubules are wholly intranuclear, the nuclear division of T. annulata is categorised, in this investigation, as closed mitosis with intranuclear spindles after the classification of Dodge and Vickerman (1980). The lack of chromosomal uncoiling and condensation observed in this study is similarly manifested in Achyla bisexualis (Griffin, Timberlake and Cheney, 1974) in which this phenomenon, at mitosis, is the result of a short nuclear cycle time, i.e. less than 51 minutes, suggesting

that it may be inefficient, and perhaps impossible, to orchestrate chromatin condensation in such a short time.

It has been demonstrated by acid phosphatase ultracytochemistry that while T. annulata trophozoites do not fuse with host lysosomes (Figures 32, 33 and 34), the dead parasites readily form "phago-lysosomes", the event which culminates in lysosomal degranulation with trophozoite digestion. The mechanism by which the living trophozoites evade fusion with host lymphocyte lysosomal granules has not been determined but is thought to be attributable to the changes in parasite membrane characteristics. In Micrococcus lysodiekticus, it has been observed that the cell walls of the lysozyme-resistant strain contain more than one hundred-fold greater content of O-acyl groups than the cell walls of the sensitive strain (Brumfitt and Wardlaw, 1958). Paper chromatography undertaken by Burness and King (1958) identified the acyl groups as being principally acetyl. Brumfitt and Wardlaw (1958) demonstrated that the lysozyme-resistant strains of M. lysodiekticus were able to acetylate certain cell wall hydroxyl groups which normally combine with lysozyme substrate. The groups, however, remained free in the sensitive strain. Virulent strains of Mycobacterium tuberculosis contain a group of strongly acidic glycolipid sulphates whereas the avirulent M. tuberculosis and most saprophytic mycobacteria do not(Goren, Brokl, Roller, Fales and Das, 1976). The catalytic activity of lysozyme is pH-dependent (Stryer, 1981) so that while it is optimal at pH 5, it drops sharply on either side of this optimal pH. There is evidence that resistance of Leishmania mexicana amazonensis to lysosomal enzyme degradation is attributable to surface antigenic

glycoproteins (Chang, 1983). In the case of T. annulata trophozoites, it is suggested in this study that structural changes accompanying the dedifferentiation of the sporozoites and their transformation to the trophozoites could affect the pellicular characteristics significantly to abrogate lysosomal fusion. The observation made in this study that acid phosphatase-positive deposits were associated with nuclear membrane and rough endoplasmic reticulum (Figure 33) demonstrates that intracellular T. annulata parasites synthesise their lysosomal enzymes.

CHAPTER FIVE

INVASION AND INTRACELLULAR DEVELOPMENT OF *T. ANNULATA* SPOROZOITES IN LYMPHOBLASTOID CELL LINES TRANSFORMED BY *T. ANNULATA* (HISSAR), *T. ANNULATA* (ANKARA) AND *T. PARVA* (MUGUGA)

CHAPTER FIVE

INVASION AND INTRACELLULAR DEVELOPMENT OF T. ANNULATA SPOROZOITES IN LYMPHOBLASTOID CELL LINES TRANSFORMED BY T. ANNULATA (HISSAR), T. ANNULATA (ANKARA) AND T. PARVA (MUGUGA)

5.1 Introduction

The observations made in this study that T. annulata sporozoites consistently attach base-first to the target bovine peripheral blood lymphocytes and that tryptic proteolysis markedly precludes attachment and interiorisation of the parasite not only indicate that attachment is receptor-dependent, but also that susceptibility is a function of the receptors. Bovine lymphoid cells infected by T. annulata (Hulliger, 1965) or T. parva (Malmquist et al., 1970) have been shown to undergo marked blastoid transformation. Such transformed cell lines lack demonstrable parasite-specific surface antigen (Duffus et al., 1978; Creemers, 1982), but they possess transformation antigens on their surfaces (Duffus et al., 1978; Spooner and Brown, 1980) and also induce autologous mixed lymphocyte reaction, MLR (Pearson, Lundin, Dolan and Stagg, 1979). Whether or not the intracellular presence of Theileria parasites leads to loss of susceptibility of transformed lymphoid cell or its ability to support the establishment of a fresh infection has not been investigated.

In this chapter, a study has been undertaken to determine (a) the effect of prior infection on target cell susceptibility to superinfection with homologous or heterologous Theileria species, (b) whether the superinfecting sporozoites develop into schizonts

and, (c) whether there is cross-susceptibility of transformed target cells.

5.2 Materials and methods

PBL were separated from defibrinated blood by the Ficoll/sodium diatrizoate gradient technique and the concentration adjusted to 4×10^6 cells/ml of complete RPMI 1640. GUTS of the Hissar strain of T. annulata prepared by grinding up 3-day fed, surface-sterilised, infective H. anatolicum anatolicum ticks, was centrifuged at 100 xg for five minutes (1,000 rpm, MSE Minor) and the supernate filtered through a sterile 25 mm Millipore Swinnex filter holder, AP25 prefilter and 8 μm filter. The filtrate of sporozoites was diluted and used at a concentration equivalent to 2 ticks/ml of MEM/3.5% BPA. Three Theileria-transformed cell lines established and maintained as detailed in Chapter 3 were used in this study. While T. parva (Muguga) cell line (passage 20) was used at 2×10^5 cells/ml, T. annulata (Ankara) and T. annulata (Hissar) cell lines (passages 11 and 8 respectively) were each used at 1×10^5 cells/ml. Infection of PBL and superinfection of each of the three transformed cell lines were carried out in 2-cm^2 wells of multi-well cluster plates in which monolayer cultures of lung fibroblastic cell line, IMR31, had been established 2-3 days previously. One ml, each, of PBL, T. annulata (Ankara), T. annulata (Hissar) and T. parva (Muguga) cell line suspensions were mixed, in wells, with 1 ml T. annulata (Hissar) GUTS filtrate. Four infected replicates of PBL and of each of the transformed cell lines were set up. For PBL and each of the lymphoblastoid cell lines, control cultures consisting of 1 ml

of the cell suspension and 1 ml MEM/BPA were set up, also in four replicates. The above groups will be referred to as follows: infected PBL (A1), control PBL (A3), superinfected T. annulata (Hissar) (B4), control T. annulata (Hissar) (B6), superinfected T. annulata (Ankara) (C7), control T. annulata (Ankara) (C9), superinfected T. parva (Muguga) (D10), and control T. parva (Muguga) (D12). The plates with cultures were immediately placed in a humidifed box, gassed with 5% CO_2/95% air and incubated at 37°C for two hours. At the end of the incubation period, cultures were pipetted out into sterile universal bottles, mixed with 20 ml complete RPMI 1640 prewarmed to 37°C and, centrifuged at 400 xg for five minutes at 20°C (1,800 rpm, MSE Chilspin). The pellets were resuspended in 1 ml fresh, complete RPMI 1640 prewarmed to 37°C, and cytocentrifuge smears prepared from 50-µl aliquots of culture suspension. These smears were stained with Giemsa stain and used for the quantitation of interiorised sporozoites. The remainder of the cultures were returned to wells and the plates placed in a humidified chamber, gassed with 5% CO_2/95% air and incubated at 37°C.

On Day 1, each culture received 1 ml fresh complete RPMI 1640. The plates were returned to a humidified chamber, gassed and incubated as above. On Day 3, 1 ml cell-free culture supernate was carefully pipetted out from each culture, and replaced with 1 ml fresh complete RPMI 1640. Cultures were then thoroughly mixed by gentle pipetting after which samples were taken for (a) mean schizont nuclear number and (b) total cell number determinations. For mean schizont nuclear number counts, cytocentrifuge smears were air-dried, fixed in technical methanol and air-dried once more. The dried

smears were hydrolysed for six minutes in NHCl pre-heated to, and maintained at, 60°C in a water bath as detailed in Chapter 3, washed three times in Giemsa buffer, pH 7.2 and stained with Giemsa stain. Schizont nuclear number counts were made on 50 infected cells in each replicate culture. Cell counts were undertaken using a haemocytometer (New Improved Neubauer) according to Schalm et al. (1975) as detailed in Chapter 3 for culture suspensions. 0.1 ml of a thoroughly mixed culture suspension was mixed with an equal amount of a 0.2% trypan blue solution in a bijou bottle and allowed to stand for five minutes. The contents of the bijou bottle were mixed by pipetting and an aliquot counted in a haemocytometer. Cell numbers were determined as described in Chapter 3.

Immediately after samples were obtained for smears and cell counts, plates with cultures were replaced in a humidified box, gassed with 5% CO_2/95% air and incubated at 37°C. On Day 6, 1 ml culture supernatant was pipetted out from infected and control PBL cultures, discarded and replaced with 1 ml fresh complete RPMI 1640 pre-warmed to 37°C. All cultures were then mixed by pipetting and samples obtained for schizont nuclear determination and cell counts. Following sampling, cultures set up using cell lines transformed by T. annulata (Ankara), T. annulata (Hissar) and T. parva (Muguga) including their controls were transferred and maintained in disposable plastic flasks of 25 cm^2 surface area (Nunc, Roskilde, Denmark) according to the technique described by Brown (1979a). They were passaged at a ratio of 1:1 (i.e. 2 ml cell suspension from a well transferred to a fresh, sterile flask containing 2 ml fresh complete growth medium), gassed with 5% CO_2/95% air and incubated at 37°C. Plates containing

infected and control PBL cultures were returned to a humidified box, gassed as above and incubated at 37°C. On Day 9, while 1 ml cell-free supernate was discarded and replaced by an equal amount of complete growth medium in both infected and control PBL cultures, transformed cell line cultures were centrifuged at 400 xg for five minutes (1,800 rpm, MSE Chilspin at 20°C), resuspended in 5 ml complete growth medium. All the cultures were sampled for cytocentrifuge smears and cell counts before gassing and incubation. On Day 12, all cultures were mixed by pipetting up and down and sampled for cell number determinations. The rest of the cell suspension material, in each replicate culture, was used for isoenzyme analysis.

The detection of glucose phosphate isomerase (GPI) isoenzyme patterns was undertaken by thin layer starch gel electrophoresis according to the technique described by Melrose, Brown and Sharma (1980). Replicate cultures of each group were pooled into a universal bottle and centrifuged at 1,500 xg for ten minutes (2,000 rpm, MSE Mistral 2L at 5°C). The pellets were resuspended in phosphate buffered saline Dulbecco A, PBS, pH 7.3 and centrifuged at 1,500 xg for ten minutes as above. This procedure was repeated three times after which the final supernatant PBS was carefully pipetted out and a volume of enzyme protectant (Kilgour and Godfrey, 1973) equal to the volume of the packed cells added. The washed cells were subjected to three cycles of freeze-thawing in a solid carbon dioxide methanol bath and centrifuged at 20,000 xg for 30 minutes at 5°C (16,000 rpm; super speed unit; MSE Mistral 2L). The resultant lysates were stored at -80°C, until required, in a solid CO_2 refrigerator, and were used undiluted for electrophoresis.

The electrophoresis was carried out on starch gels prepared by adding 4.25 g of hydrolysed starch to 40 ml of gel buffer prepared by diluting 1.5 ml of 0.272M potassium phosphate buffer (Shaw and Koen, 1968) with 38.5 ml distilled water. The resultant starch suspension was gently heated until it became less viscous when it was degassed and spread on a glass plate, 230 mm x 150 mm, as described by Wraxall and Culliford (1968). The gels were used for electrophoresis 30 minutes after spreading. The origin was placed 9.0 cm from the cathode end. Slots, approximately 1 cm long, were cut in the gel using a gel slot meter (Shandon Southern Instruments Ltd.) and the lysates were applied on single 1 cm strands of washed white cotton thread. When the parasite enzyme activity was weak, two strands were placed in each slot. Electrophoresis was carried out at 8°C using a potential difference of 350 volts (23.3 volts/cm; Vokam 500-150 power supply; Shandon Southern Instruments Ltd.) for three hours and an initial current of about 20 mA per gel in a Shandon 600 x 100 chamber.

After electrophoresis a plastic mould (11.7 cm x 11.7 cm) was positioned on the gel towards the anode, firmly and gently, to form a seal. Bands of GPI activity were located using two developers, (a) and (b). Both contained 3 ml 0.3M Tris-HCl, pH 8.0, 3 ml distilled water; 1 ml 3-(4,5-dimethylthiazolyl-2)-2,5 diphenyl tetrazolium bromide, MTT, solution (4 mg/ml); 30 µl glucose-6-phosphate dehydrogenase, G-6-PDH, (Sigma type XI, Sigma Chemical Co.) (265 u/ml); 1 ml 0.1M $MgCl_2$; 0.5 ml NADP (6 mg/ml); 0.5 ml fructose-6-phosphate (5 mg/ml). In addition developer (a) contained approximately 2-3 mg of an intermediate electron carrier, 5-methyl-phenazinium

methylsulphate (phenazine methosulphate, PMS) (Wilkinson, 1970) which in developer (b) was replaced by 0.1 ml meldola blue (6 mg/ml) (all the chemicals from Sigma Chemical Co.). Approximately 70 mg purified agar (Oxoid L28, Oxoid Ltd.) were weighed out and dissolved in 6 ml distilled water by heating to boiling. The solution was cooled to approximately 50°C and then added to the developer solution and mixed by gentle inversion to minimise bubble formation. The agar overlay was poured into the plastic mould resting on top of the gel and the complete plate tilted to spread the solution. The overlay was allowed to set while the gel was still on the cooling plate after which the gel was put in an incubator at 37°C for one hour. If the enzyme activity was weak, the incubation time was increased to two hours. Gels were photographed with a Polaroid MP3 camera using type 55 film, and a Kodak 16 yellow filter (Kodak Ltd., Hemel Hempstead, Hertfordshire).

5.3 Results

Figure 36 shows T. annulata (Hissar) sporozoites attached to the plasma membrane of, and within, schizont-containing transformed cells as observed in B4 and C7. The mean numbers of interiorised T. annulata (Hissar) sporozoites counted within PBL (A1), and cell lines transformed by T. annulata (Hissar) (B4), T. annulata (Ankara) (C7) and T. parva (Muguga) (D10) are presented in Table 6. Although B4 and C7 were susceptible to superinfection by T. annulata (Hissar) sporozoites, D10 was found to be refractory so that neither attachment nor invasion was detected.

Figure 36 A Giemsa-stained, cytocentrifuge smear of schizont-containing T. annulata-transformed lymphoblasts superinfected with T. annulata (Hissar) sporozoites. Sporozoites are depicted free, attached to the cell surface, and intracellular. The incumbent schizont shows a mass of a more basophilic cytoplasm and larger nuclei than those of extracellular and freshly interiorised sporozoites. Magnification, x 1,280

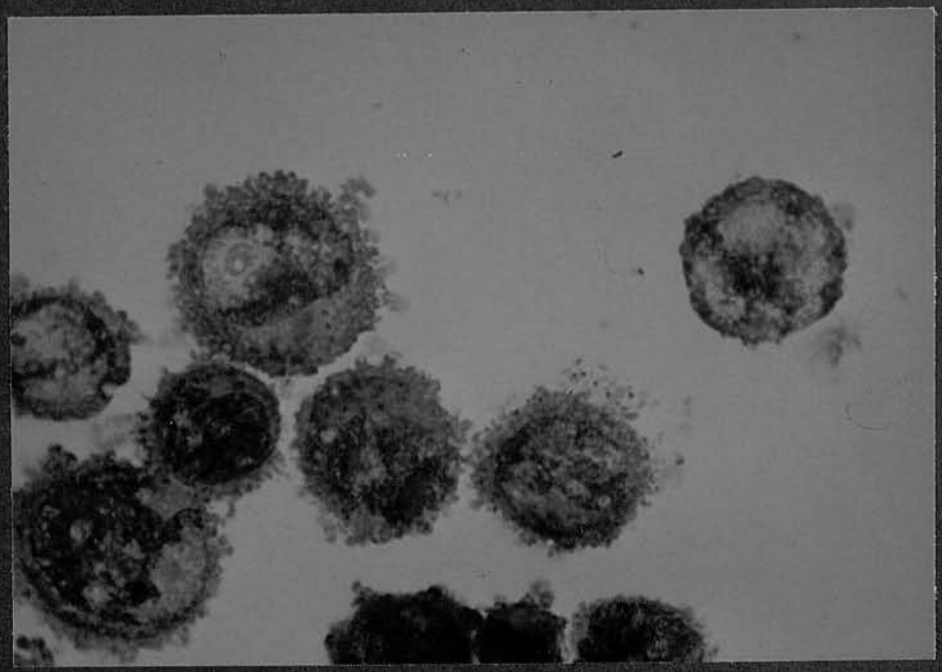


Table 6 The number of T. annulata (Hissar) sporozoites infecting PBL, and superinfecting lymphoblastoid cell lines transformed by T. annulata and T. parva per 1,000 lymphoid cells

Cell line n = 4	Means and standard deviations
A1 - PBL	226.8 ± 10.05
B4 - T. annulata (Hissar)	355.0 ± 168.73
C7 - T. annulata (Ankara)	78.8 ± 39.68
D10 - T. parva (Muguga)	0.0 ± 0.00

Comparisons, by t-test, of the mean cell numbers between A1 and A3, B4 and B6, C7 and C9, and D10 and D12 are presented in Tables 7-10. Detailed data are presented in Appendix Table 2. Table 7 shows that the mean numbers of cells in the infected PBL (A1) on Days 9 and 12 are highly significantly greater than in the non-infected counterpart (A3). A comparison between B4 and B6 (Table 8) revealed that, apart from the odd significant difference observed on Day 3, the mean cell numbers in the two groups remained consistently similar. No significant differences were observed between C7 and C9 (Table 9). Comparison between the two groups of T. parva (Muguga) cell line, D10 and D12, revealed that apart from the significant difference observed on Day 6, the mean cell numbers were similar on Days 3, 9 and 12 (Table 10).

Table 7 Comparison of mean cell numbers (x 10^5 except Day 6 (x 10^6)) between PBL control (A1) and PBL infected with T. annulata sporozoites on Days 3, 6, 9 and 12 in culture

Day	Treatment	Means and standard deviations	$t_{(6)}$
3	A1	3.00 ± 0.52	0.342
	A3	2.88 ± 0.47	
6	A1	1.19 ± 0.18	0.652
	A3	1.13 ± 0.05	
9	A1	6.78 ± 1.28	6.191***
	A3	2.73 ± 0.26	
12	A1	7.33 - 1.35	8.637***
	A3	1.28 - 0.36	

***P < 0.001

Table 8 Comparison of mean cell numbers (x 10^6 except Day 3 (x 10^5)) between B4 and B6 on Days 3, 6, 9 and 12 in culture

Day	Treatment	Means and standard deviations	$t_{(6)}$
3	B4	2.60 ± 0.20	3.182*
	B6	2.13 ± 0.22	
6	B4	1.88 ± 0.26	0.241
	B6	1.93 ± 0.32	
9	B4	2.43 ± 0.22	0.255
	B6	2.35 ± 0.54	
12	B4	3.95 ± 0.53	0.764
	B6	3.48 ± 1.13	

*P < 0.05

Table 9 Comparison of mean cell numbers (x 10^6 except Day 3 (x 10^5)) between C7 and C9 on Days 3, 6, 9 and 12 in culture

Day	Treatment	Means and standard deviations	$t_{(6)}$
3	C7	1.65 ± 0.21	0.128
	C9	1.63 ± 0.33	
6	C7	1.63 ± 0.39	0.000
	C9	1.63 ± 0.24	
9	C7	2.35 ± 0.72	0.137
	C9	2.40 ± 0.14	
12	C7	3.00 ± 0.67	1.163
	C9	2.60 ± 0.16	

Table 10 Comparison of mean cell numbers (x10^6 except Day 3 (x 10^5)) between D10 and D12 on Days 3, 6, 9 and 12$^+$ in culture

Day	Treatment	Means and standard deviations	$t_{(6)}$
3	D10	3.38 ± 1.09	1.132
	D12	2.75 ± 0.17	
6	D10	4.23 ± 0.15	3.289*
	D12	2.85 ± 0.82	
9	D10	5.45 ± 0.53	0.294
	D12	5.18 ± 1.80	
12	D10	0.90 ± 0.20	2.16
	D12	1.13 ± 0.05	

*$P < 0.05$ $^+$(x 10^7)

Day means of schizont nuclear numbers, determined in 50 infected cells per replicate culture for treatments A1, B4, B6, C7, C9, D10 and D12 are presented in Table 11 and detailed data in Appendix Table 3. A two-way analysis of variance of treatments B4, B6, C7, C9, D10 and D12 and days revealed significant differences between treatments ($F^{5}_{54} = 29.37$; $P < 0.01$) and between days ($F^{2}_{54} = 16.02$; $P < 0.01$) but the interaction was not significant ($F^{10}_{54} = 0.45$; $P > 0.05$). The difference between treatments was resolved when treatments D10 and D12 were omitted from the analysis ($F^{3}_{36} = 0.46$; $P > 0.05$). The differences between days, however, remained ($F^{2}_{36} = 13.42$; $P < 0.01$). Reanalysis as a one-way anova confirmed the significance of the difference between days ($F^{2}_{45} = 15.28$; $P < 0.01$) and further analysis by Duncan's multiple range test showed that the day means fell into two significant subsets, viz. day (6) and days (9, 3).

Table 11 Day means of schizont nuclear numbers in freshly infected PBL (A1), superinfected T. annulata (Hissar) (B4), T. annulata (Hissar) control (B6), superinfected T. annulata (Ankara) (C7), T. annulata (Ankara) control (C9), inoculated T. parva (D10) and T. parva control (D12) per 50 infected cells

Cell line n = 4	Means and standard deviations Day 3	Day 6	Day 9
A1	6.60 ± 0.80	9.68 ± 0.85	14.58 ± 1.58
B4	14.65 ± 2.35	12.53 ± 0.61	13.93 ± 1.00
B6	14.15 ± 0.53	12.05 ± 1.03	13.33 ± 0.94
C7	13.80 ± 0.37	12.15 ± 0.95	14.25 ± 0.72
C9	13.80 ± 0.24	12.25 ± 1.33	14.03 ± 1.49
D10	10.85 ± 0.66	9.05 ± 0.78	10.40 ± 1.61
D12	10.70 ± 1.42	9.45 ± 1.44	9.70 ± 0.50

When the D10 and D12 data were analysed together as a two-way anova, the difference attributable to treatments was not significant ($F^{1}_{18} = 0.10$). Reanalysis as a one-way anova revealed that differences attributable to the days were significant ($F^{2}_{21} = 3.88$; $P < 0.05$) and further analysis by Duncan's multiple range test showed that the day means fell into two overlapping subsets viz. (6, 9) and (9, 3).

Because differences attributable to days were significant, the treatment data were reanalysed on each day and significant subsets between treatments determined (Table 12).

Table 12 Comparison of the day means of schizont nuclear numbers

Day	Ranked Means: Low → High		
3	A	D	B/C
6	D	A	B/C
9	D	B/C	A

⌊⌋ = Significant subset

A comparison of glucose phosphate isomerase (GPI) isoenzyme patterns of lysates from non-infected bovine PBL and the lymphoblastoid cell line groups A1, B4, B6, C7, C9, D10 and D12 is presented in Figure 37. The bands of host cell GPI activity in all the treatments were identical and localised around the origin. Bands of the isoenzyme activity attributable to the parasites moved more anodally, T. annulata bands migrating farther towards the anode than those of T. parva. Differences were not only demonstrated between the two Theileria species but also between the two strains of T.

Figure 37 Glucose phosphate isomerase (GPI) isoenzyme patterns of lysates of non-infected bovine PBL (P), and the lymphoblastoid cell lines A1, B4, B6, C7, C9, D10 and D12.


+
P
A1
B4
B6
C7
C9
D10
D12
P

annulata viz. T. annulata (Hissar) and T. annulata (Ankara). No superimposition of species- or strain-specific bands of GPI activity was observed. The A1 cell line produced a markedly diminutive band of host cell GPI activity and lack of parasite GPI activity.

5.4 Discussion

The observation in this study that T. annulata (Hissar) sporozoites superinfected T. annulata-transformed cell lines (Table 6) demonstrates that Theileria-dependent lymphoblastoid transformation does not produce alterations on the host cell membrane that preclude its recognition and superinfection by the same parasite species. T. parva-transformation lymphoblasts have, however, been shown to be completely refractory to superinfection by T. annulata sporozoites (Table 6). This observation shows that the cell types or subpopulations susceptible to infection and transformation by either of the two Theileria species are different and possess specific surface receptors for either parasite. Previous studies have suggested that cells transformed by T. parva share a membrane antigen with calf thymocytes (Duffus et al., 1978), represent a discrete subpopulation of T lymphocytes (Pinder et al., 1981) and appeared to belong to a minor population as determined by BoLA phenotype studies (Spooner and Brown, 1980). Controversy still exists on the characteristics of T. annulata-transformed cells. While Mahan (1980) concluded that the cells were of T sub-type, Musiime (personal communication) observed that 30-40% of T. annulata-transformed lymphoblasts were positive for Fc receptors and that they were negative for surface Ig and fluoresceinated peanut agglutinin staining, thus implicating B sub-population.

The number of T. annulata (Hissar) sporozoites counted within lymphoblasts transformed by T. annulata (Hissar) or T. annulata (Ankara) (Table 6) is surprisingly low, especially in the light of the fact that these cell lines are clones derived from selected, susceptible lymphoid cells. Although reasons for such a diminished response are not known, it is likely that superinfection may only be possible at a certain stage in the lymphoblast cycle.

Comparison of the mean cell numbers between A1 and A3 (Table 7) demonstrates that following a fresh infection of bovine peripheral blood lymphocytes by T. annulata, no significant parasite-induced changes in the number of cells are manifest until several days later when intense replication of transformed lymphoid cells leads to highly significant increases. In such a culture, the mean nuclear number of freshly established T. annulata schizonts has been shown, in this study, to undergo a steady increase (Table 12) and equalize those in long standing cultures. The mean number of nuclei per schizont, however, stabilises when the rate of distribution of daughter schizonts in daughter lymphoblasts, during cell division, is constant (Hulliger, 1965).

Although the mean schizont nuclear number in treatment A1 was initially shown to undergo a steady increase, analysis of similar data in groups B and C in which superinfection was demonstrated revealed no significant differences between the treatments inoculated with T. annulata (Hissar) sporozoites, viz. B4 and C7, and their respective control counterparts, B6 and C9. This observation implies that the parasites which invaded the above already established, schizont-containing cell lines failed to develop, an observation

which is confirmed in this study by the formation of uncontaminated species- or strain-specific bands of glucose phosphate isomerase isoenzyme activity (Figure 37). Isoenzymes of glucose phosphate isomerase have been shown to differentiate T. parva from T. annulata (Musisi, 1978) and to tell apart intraspecific differences between the Hissar and Ankara strains of T. annulata (Melrose et al., 1980). The much smaller number of cells in treatment A1 led to the development of a markedly faint host cell GPI activity, and to lack of demonstrable parasite GPI isoenzyme bands (Figure 37). The superinfected, schizont-containing cell lines could not have been similarly affected since the comparison of the mean cell number data (Tables 8-10) revealed no significant losses.

The inability of the superinfecting T. annulata sporozoites to develop within transformed, schizont-bearing cells implicates the existence of a schizont-induced interference phenomenon operating within Theileria-transformed lymphoblasts. The phenomenon may operate in several ways: (a) alter host cell surface, (b) competitively inhibit units of replicative machinery or (c) alter host cell metabolic pathways so that they are unavailable for the superinfecting sporozoites. Alteration of the host cell surface seems an unlikely mechanism in T. annulata infection since it has been demonstrated in this study that transformed cell lines (B4 and C7; Table 6) were superinfected. It is suggested that the incumbent schizont may interfere with the establishment of the superinfecting sporozoites either by competition for units of replicative machinery such as gene sites or through the alteration of host cell metabolic pathways such as glycosylation.

CHAPTER SIX

DEMONSTRATION OF ENERGY METABOLIC PATHWAYS IN T. ANNULATA SPOROZOITES AND THEIR IMPORTANCE IN THE INVASION OF PBL BY THE SPOROZOITES

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6.3 Ultracytochemistry of Marker enzymes

6.3.1 Introduction

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(a) Introduction

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(a) Introduction

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CHAPTER SIX

DEMONSTRATION OF ENERGY METABOLIC PATHWAYS IN T. ANNULATA SPOROZOITES AND THEIR IMPORTANCE IN THE INVASION OF PBL BY THE SPOROZOITES

6.1 Introduction

In Chapter 4 of this thesis it was shown that invasion of bovine peripheral blood lymphocytes by T. annulata is actively accomplished by the sporozoites. This not only implies the existence, within the parasite, of pathways for the generation of metabolic energy but also suggests that the pathways are intact and functional so that the sporozoites independently generate the energy needed for their movement and invasive activity. That metabolic pathways exist in the salivary gland stages of T. annulata has been demonstrated (Weber, 1980; 1982) but the role they play in the activities of the extracellular sporozoite has not been investigated. The use of chemicals that are known to selectively inhibit glycolysis, various steps in the mitochondrial respiratory chain and adenosine triphosphate (ATP) formation from adenosine diphosphate (ADP) and inorganic phosphate would pinpoint which pathways are present in the infective T. annulata sporozoites and demonstrate their role during invasion of PBL by the sporozoites.

In this chapter, both selective metabolic pathway inhibition and ultracytochemical demonstration of the respective marker enzymes, viz. lactic dehydrogenase, succinic dehydrogenase and cytochrome oxidase, have been undertaken.

6.2 Effect of treating T. annulata sporozoites with specific energy metabolic pathway inhibitors on their ability to invade PBL

6.2.1 Inhibitors of glycolysis

(a) Lithium iodoacetate

(i) Introduction - Iodoacetate inhibits the enzyme, glyceraldehyde-3-phosphate dehydrogenase, which catalyses the conversion of glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate (Bowman and Rand, 1980). Glyceraldehyde-3-phosphate dehydrogenase is an -SH enzyme that forms a thio-ester intermediate with the substrate. In the presence of iodoacetate the thiol groups of cysteine and the secondary nitrogen in the imidazole ring of histidine are alkylated, glycolysis stops with the formation of triose phosphates, and the production of ATP is severely depressed since in the next step, the high phosphoryl transfer potential of 1,3-diphosphoglycerate, is normally used to generate ATP (Stryer, 1981). In this section, iodoacetate has been used as a blocking agent in an investigation to determine the significance of glycolysis as a source of energy for T. annulata sporozoite activity.

(ii) Materials and methods - For iodoacetate and other metabolic inhibitors used in Section 6.2, PBL were separated from buffy coat obtained from defibrinated blood on a Ficoll/sodium diatrizoate gradient as previously described. The concentration was adjusted to 8×10^6 cells/ml of complete RPMI 1640. T. annulata GUTS used in each metabolic energy inhibition experiment was prepared by grinding up 40 infective, 3-day fed H. anatolicum anatolicum

in MEM/3.5% BPA, centrifuging at 100 xg for ten minutes to give a sporozoite supernate, and filtering the supernate through a sterile 8 μm membrane filter. The filtrate containing T. annulata sporozoites was used at a concentration equivalent to 2 ticks/ml MEM/3.5% BPA.

Each metabolic energy inhibitor drug used in Section 6.2 was prepared at twice its desired molar concentration in Eagle's MEM with Earle's salts containing 20% foetal calf serum, antibiotics and 2 mM/ml L-glutamine for the ultimate 1:1 mixing with T. annulata sporozoite suspensions. For iodoacetate (lithium salt; molecular weight 191.8; CalBiochem-Behring Corp., La Jolla, California, CA 92037) a 2×10^{-2}M solution was prepared by dissolving 38.36 mg of the salt in 10 ml of medium which was then serially diluted to give 2×10^{-3}M, 2×10^{-4}M, 2×10^{-5}M and 2×10^{-6}M solutions.

The treatment of T. annulata GUTS filtrate with metabolic energy inhibitor drugs and the establishment of cultures, thereafter, using the treated sporozoites were undertaken as detailed below for lithium iodoacetate. To 0.25 ml of T. annulata GUTS, 0.25 ml of the appropriate molar concentration of lithium iodoacetate solution was added and the mixtures incubated at 37°C for three hours. Cultures were established in multi-well cluster plates by mixing iodoacetate-treated sporozoite suspensions with 0.25 ml of lymphocyte suspension. Four replicate cultures were set up per each molar concentration of iodoacetate and untreated controls. Two groups of control cultures were set up for iodoacetate but only one for the other inhibitors. In one control group, PBL were inoculated with T. annulata sporozoites which had been pre-

incubated at 37°C for three hours while in the second set of control cultures, PBL were inoculated with sporozoites which had initially been kept at 4°C but were allowed to stand at 20°C before setting up cultures. It is this second group which was eliminated from the other inhibition experiments undertaken below. In both groups, 0.25 ml GUTS were mixed with 0.25 ml PBL suspension. Plates containing cultures were placed in a humidified box, gassed with 5% CO_2/95% air and incubated at 37°C for one hour. At the end of the incubation period, cultures were thoroughly mixed and 50-µl aliquots obtained from each replicate culture for cytocentrifuge smears stained with Giemsa stain for assessment of iodoacetate activity. For iodoacetate and other metabolic energy inhibitor drugs used below, assessment was made by determining the number of T. annulata sporozoites which had invaded and entered target cells per 1000 lymphoid cells.

(b) Sodium fluoride

(i) Introduction - Fluoride ions disrupt glycolysis by inhibiting the enzyme enolase (Bowman and Rand, 1980) which catalyses the formation of phosphoenolpyruvate from 2-phosphoglycerate. The inhibition abrogates the next step in which conversion of phosphoenolpyruvate to pyruvate is accompanied by the transfer of a phosphoryl group from the former compound to ADP to generate ATP. The significance of glycolysis as a source of energy for T. annulata sporozoite activity has been investigated here by using sodium fluoride as a specific inhibitor.

(ii) Materials and methods - PBL and GUTS of T. annulata sporozoites were prepared as previously described for iodoacetate. A 2 x 10^{-1}M solution of sodium fluoride (molecular weight 42; Sigma Chemical Company, St. Louis, Missouri) was prepared by dissolving 84 mg of the salt in 10 ml of medium which was then serially diluted at $\log_{10}$ to provide 2 x 10^{-2}M, 2 x 10^{-3}M and 2 x 10^{-4}M solutions.

Treatment of T. annulata sporozoites with appropriate molar concentrations of sodium fluoride, the establishment of cultures with treated sporozoites thereafter, the sampling of cultures and their assessment for sodium fluoride activity were carried out as detailed for lithium iodoacetate.

6.2.2 Inhibitors of the respiratory chain

(a) Potassium cyanide

(i) Introduction - The cyanide ion inhibits the electron flow between the terminal members of the respiratory chain, the cytochromes a and a_3 complex, and O_2 by reacting with the ferric (Fe^{3+}) form in the iron-sulphur (FeS) central core of the cytochromes a and a_3 complex (Stryer, 1981). Cyanide inhibition has the same effect as complete lack of oxygen and all the components of the respiratory chain are in a reduced state so that no ATP can be generated. In this section, an investigation has been undertaken to determine the effect cyanide inhibition of the mitochondrial respiratory chain of T. annulata sporozoites would have on their invasive activity.

(ii) Materials and methods - PBL and GUTS of T. annulata sporozoites were prepared as described earlier. A 2 x 10^{-2}M solution of potassium cyanide (Analar grade; molecular weight 65.12; BDH Chemicals Ltd., Broom Road, Poole, England) was prepared by dissolving 13.0 mg of the salt in 10 ml of medium which was then serially diluted at $\log_{10}$ to give 2 x 10^{-3}M, 2 x 10^{-4}M, 2 x 10^{5}M and 2 x 10^{-6}M solutions.

Treatment of T. annulata sporozoites with the above molar concentrations of potassium cyanide, the establishment of cultures with the treated sporozoites, the sampling of cultures and their assessment for potassium cyanide activity were undertaken using methods similar to those described before.

(b) Antimycin A

(i) Introduction - The effect of antimycin A is localised between cytochromes b and c_1, where the antibiotic inhibits electron flow, thus preventing ATP synthesis coupled to generation of a proton gradient at coenzyme QH_2 - cytochrome c reductase complex (Potter and Reif, 1952; Klein and Hartree, 1955; Racker, 1976; Boyer, Chance, Ernster, Mitchell, Racker and Slater, 1977; Stryer, 1981).

In this section, the antibiotic has been employed in an investigation to determine the importance of the respiratory chain as a source of energy for T. annulata sporozoite activity.

(ii) Materials and methods - Details pertinent to the preparation of PBL and T. annulata sporozoites have been provided before. A 2 x 10^{-3}M solution of antimycin A (molecular weight 548.6;

Calbiochem-Behring Corp.) was prepared by dissolving 10.0 mg of the antibiotic powder in 9.11 mg of medium which was then serially diluted to give 2 x 10^{-4}M, 2 x 10^{-5}M and 2 x 10^{-6}M solutions.

Incubation of T. annulata sporozoites with the above concentrations of antimycin A, setting up of cultures with the treated sporozoites, the sampling of cultures and their assessment for antimycin A activity were carried out as described for lithium iodoacetate.

6.2.3 Inhibition of oxidative phosphorylation by 2,4-dinitrophenol

(a) Introduction

Oxidative phosphorylation has been comprehensively discussed by Stryer (1981). It is the process in which ATP is formed as electrons are transferred, between the members of the respiratory chain, from NADH or $FADH_2$ to O_2 by a series of electron carriers. The step-by-step transfer of electrons from NADH or $FADH_2$ to O_2 through the numerous electron carriers located in the inner mitochondrial membrane leads to the pumping of protons out of the mitochondrial matrix and to the generation of a membrane potential (proton-motive force). Protons are pumped by three kinds of electron-transfer complexes: (1) NADH-Q reductase complex; (2) QH_2-cytochrome c reductase complex; and (3) the cytochrome c oxidase complex. The flow of electrons through each of these three complexes generates a proton gradient sufficient to synthesise one molecule of ATP. The synthesis of ATP takes place when protons flow back to the mitochondrial matrix through a molecular assembly in the inner mitochondrial membrane, the mitochondrial ATPase. Thus, oxidation and phosphorylation are coupled by a proton gradient

across the inner mitochondrial membrane.

2,4-dinitrophenol disrupts this tight coupling of electron transport and phosphorylation by dissipating the proton gradient. In its presence, the electron transport from NADH or $FADH_2$ to O_2 proceeds normally, but ATP is not formed by the mitochondrial ATPase because the proton-motive force across the inner mitochondrial membrane is dissipated.

In this section, 2,4-dinitrophenol has been used in a study to determine the existence of oxidative phosphorylation in T. annulata sporozoites and to confirm the belief that ATP is the high energy intermediate utilised in energy-dependent lymphocyte invasion by the sporozoites.

(b) Materials and methods

Details pertinent to the preparation of PBL and T. annulata GUTS have been provided previously. A 2×10^{-2}M solution of 2,4-dinitrophenol (anhydrous molecular weight 184.1; Sigma Chemical Co.) was prepared by dissolving 36.82 mg of the compound in 10 ml of medium and then serially diluted to give 2×10^{-3}M, 2×10^{-4}M, 2×10^{-5}M and 2×10^{-6}M solutions.

Treatment of T. annulata sporozoites with the above molar concentrations of 2,4-dinitrophenol, establishment of cultures with the treated sporozoites thereafter, the sampling of cultures and their assessment for 2,4-dinitrophenol activity were carried out as described earlier.

6.2.4 Results

Comparison, by t-test (Table 13), of data obtained from cultures set up with T. annulata sporozoites pre-warmed to, and maintained at, 37°C for three hours (C^2) and those established from sporozoites initially kept at 4°C but allowed to stand at 20°C (C^1) before mixing with PBL, revealed no significant difference ($t_{(6)} = 0.267$; $P > 0.05$) indicating that the incubation of sporozoites at 37°C for three hours did not affect their infectivity.

Linear regression analysis for all the specific metabolic pathway inhibitors, except antimycin A (significant at $P < 0.05 > 0.01$), revealed highly significant correlation corefficients ($P < 0.001$) depicting an inverse relationship between the dose of inhibitor and the number of T. annulata sporozoites interiorised per 1,000 lymphocytes (Table 14; and detailed data in Appendix Table 4). Variance ratio tests revealed highly significant regressions ($P < 0.01$) for all inhibitors, but significant deviations from regression were also revealed for lithium iodoacetate ($F = 5.69$; $P < 0.05$) and sodium fluoride ($F = 22.73$; $P < 0.05$). The above observations depicted a linear relationship between the dose of inhibitor and the number of interiorised T. annulata sporozoites for all the reagents used in this investigation except for lithium iodoacetate and sodium fluoride ions where a curvilinear relationship was depicted.

Table 13 Comparison of the mean number of sporozoites/1,000 lymphocytes in the two control groups (C^1 and C^2) for lithium iodoacetate experiment

Group	Number of observations	Means and standard deviations	t-value
C^1	4	702.3 ± 119.52	$t_{(6)} = 0.267$*
C^2	4	677.5 ± 141.99	

*P > 0.05 (not significant)

C^1 - Set up by mixing T. annulata sporozoites, kept at 4°C and warmed to 20°C, with PBL

C^2 - Set up by mixing T. annulata sporozoites, pre-warmed to 37°C for three hours, with PBL

Table 14 Relationship between the metabolic inhibitor drug doses and the number of interiorised T. annulata sporozoites

Reagent	Number of observations	Correlation coefficient	Variance ratios	
			Regression	Deviation from regression
Lithium iodoacetate	20	0.812***	138.30**	5.69*
Sodium fluoride	12	0.858***	191.64**	22.73*
Potassium cyanide	20	0.758***	65.71**	1.98
Antimycin A	16	0.611*	19.39**	0.18
2,4-Dinitro-phenol	20	0.943***	391.76**	2.84

*P < 0.05 > 0.01

**P < 0.010 > 0.001

***P < 0.001

6.3 Ultracytochemical demonstration of marker enzymes for energy metabolic pathways in T. annulata sporozoites

6.3.1 Introduction

The basis of cytochemical demonstration of dehydrogenase enzymes has been investigated (Kerpel-Fronius and Hajos, 1968; Hanker, Kusyk, Bloom and Pearse, 1973). The method is based on a simultaneous coupling reaction in which ferricyanide, an artificial electron acceptor, produced is captured by copper at the site of the enzyme activity. Cupric ferricyanide, or Hatchett's brown, is the primary reaction product precipitated. The incorporation of an appropriate chelator, for example, tartrate, prevents the precipitation of copper by other components of the incubation medium. The localisation obtained is of high accuracy so that no nonspecific attachment of the chemical to tissue components can be detected. Upon osmication an osmium black end-product, which is ideal for light or electron microscopy, is produced. Dimethylsulfoxide (DMSO) mediates electron transfer from cysteine as well as from the reduced carriers to the artificial acceptors so that when it is omitted from the incubation medium, no reaction is obtained.

6.3.2 Lactic dehydrogenase for glycolysis

(a) Introduction

Glycolysis has both an aerobic and anaerobic mode. In the aerobic metabolism, pyruvate is oxidatively decaboxylated to acetyl-coA which can then be completely oxidised in the citric acid cycle to CO_2 and H_2O. The NADH produced during the oxidation

of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate as well as in the oxidative decaboxylation of pyruvate and in subsequent reactions of the citric acid cycle is reoxidised in the electron transport chain in the inner mitochondrial membrane. When the amount of oxygen is limiting, pyruvate enters into fermentation reactions and is reduced to lactate with reduced nicotinamide adenine dinucleotide (NADH). This, the final step of anaerobic glycolysis, is catalysed by lactic dehydrogenase. The reaction can be coupled to the NADH-producing oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. The reduction of pyruvate to lactate exactly balances the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate and sustains the continued operation of glycolysis under anaerobic conditions because the NADH formed in the oxidation of glyceraldehyde-3-phosphate is regenerated as oxidised nicotinamide adenine dinucleotide (NAD^+) in the reduction of pyruvate to lactate. If NAD^+ were not regenerated, glycolysis could not proceed beyond glyceraldehyde-3-phosphate.

(b) Materials and methods

Whole salivary glands used in all the enzyme ultracytochemistry experiments were obtained by cuticular dissection of adult H. anatolicum anatolicum ticks heavily infected with T. annulata and fed on rabbit ears for three days. The glands were submerged in ice-cold PBS, pH 7.3 (Dulbecco A) in two 50-cm diameter petri dishes, A and B. While A (used as control in all the experiments) contained salivary glands from the left side of the ticks, B comprised those from the right side. For the demonstration of

lactic dehydrogenase activity, both A and B specimens were fixed in 4% paraformaldehyde (TAAB Laboratories) in 0.1M sodium phosphate (Analar grade; BDH) buffer, pH 7.2, for 15 minutes at 4°C. They were subsequently washed in the 0.1M sodium phosphate buffer for three hours, the buffer being changed at hourly intervals. Both groups A and B specimens were incubated for 60 minutes in ferricyanide medium (Kerpel-Fronius and Hajos, 1968) as modified by Hanker et al. (1973) for the demonstration of lactic dehydrogenase. Group A (control) salivary glands were incubated in the medium from which the substrate, L-lactate, had been omitted. The medium was prepared immediately before use and was composed of: (i) 3 ml 0.5M sodium potassium-tartrate (Analar grade; BDH dissolved in 0.1M sodium phosphate buffer, pH 7.2, (ii) 0.8 ml 0.1M sodium phosphate buffer, pH 7.2. (i) and (ii) were thoroughly mixed with a magnetic stirrer after which the following remaining components of the medium were added in the order indicated, (iii) 0.35 ml 0.3M $CuSO_4$ (Analar, BDH) added dropwise, (iv) 25 mg L-lactate (Sigma Chemical Co.) dissolved in 0.025 ml of 0.3M $CuSO_4$, (v) 5 mg NAD^+ (Sigma), (vi) 1.5 ml dimethylsulfoxide (DMSO) (Analar grade; BDH). The pH of the mixture was adjusted to 6.6-6.8 and then (vii) 0.3 ml 0.05M potassium ferricyanide (Analar grade; BDH) finally added dropwise. The final pH was 6.7 The control medium comprised all the above chemicals except L-lactate. Each medium was filtered through a fast, prefolded filter paper onto the respective specimen, e.g. control medium onto A, while complete medium onto B. Following incubation, specimens were washed in 0.1M sodium phosphate buffer for 20 minutes

and then fixed in 2.5% glutaraldehyde in 0.1M sodium phosphate buffer at 4°C. Tissues were washed in three changes of 0.1M sodium phosphate buffer and fixed in 1% osmium tetroxide in 0.1M cacodylate buffer, pH 7.2 at room temperature for 1½ hours.

Osmicated and washed specimens in all the enzyme ultracytochemistry experiments were dehydrated in a series of mixtures of water and component A Durcupan embedding medium (Fluka AG, Buchs SG, Switzerland), the concentration of component A increasing in consecutive stages as recommended in Data Sheet No. 230 (Polysciences Inc., Philadelphia, USA). The steps were as detailed below:

Dehydration bath

1st tray: 50% component A with 50% water for 30 minutes, with shaking
2nd tray: 70% component A with 30% water for 45 minutes, with shaking
3rd tray: 90% component A with 10% water for 45 minutes, with shaking
4th tray: 100% component A for 90 minutes, with shaking
5th tray: 100% component A for 90 minutes, with shaking

The tissues, now water free, were processed through Durcupan/Araldite mixtures as detailed below:

Durcupan (Comp. A) (%)	Araldite I* (%)	Araldite II** (%)	Time (at 50°C)
70	30	-	1 hour
50	50	-	1 hour
30	70	-	overnight
-	100	-	1 hour
-	-	100	3 changes of 30 min. each

*Araldite I
10 ml Araldite CY 212
10 ml Hardener HY 964 (Agar Aids)

**Araldite II
Araldite I plus 2%
Accelerator BDMA (Agar Aids)

The tissues were embedded in fresh Araldite II and polymerised at 50°C for 48 hours.

Ultrathin sections of the polymerised tissue blocks in each enzyme experiment were cut with LKB glass knives on a Cambridge-Huxley Mark II ultramicrotome, mounted on 200-mesh copper grids and stained with vanadatomolybdate solution (Callahan and Horner, 1964) freshly prepared and used as detailed below.

The staining solution was prepared by mixing 20 ml of freshly prepared 1% vanadyl sulphate, pH 3.6, with 80 ml 1% ammonium molybdate (both products of Polysciences Inc.). The resultant solution, initially dark purple, finally became clear yellow and was of pH 3.2. Immediately before staining, the pH of vanadatomolybdate was adjusted to 7, to eliminate the mild tendency of the stain to precipitate at low pH, and the solution was filtered through a 0.025 µm MF filter in a 25 mm Swinnex-type adaptor (both products of Millipore Corporation) as a precautionary measure against contamination by undissolved particulate material or formed precipitate.

Grids containing sections of salivary glands of H. anatolicum anatolicum infected with T. annulata were floated, tissue surface down, on drops of vanadatomolybdate in a porcelain spot plate. The preparation was covered to exclude dust particles and avoid evaporation. The specimens were stained for 30 minutes after which the grids were rinsed by holding them in a stream of distilled water from a wash bottle. They were finally dried between sheets of bibulous paper and examined using Philips 400 electron microscope.

6.3.3 Succinic dehydrogenase for citric acid cycle

(a) Introduction

In the citric acid cycle succinate is oxidised to fumarate. The reaction is catalysed by the enzyme succinic dehydrogenase covalently bound to a prosthetic group, flavin adenine dinucleotide (FAD), which is the hydrogen acceptor in the reaction and is consequently reduced to $FADH_2$ (Bowman and Rand, 1980; Stryer, 1981). The $FADH_2$ produced by the oxidation of succinate does not dissociate from the enzyme which, unlike the other enzymes in the citric acid cycle, forms an integral part of the inner mitochondrial membrane and is directly linked to the electron-transport chain. The electrons from the $FADH_2$ are transferred directly to the FeS (Fe^{3+}) centre of the enzyme and to the coenzyme Q of the succinate-Q reductase complex for entry into the electron-transport chain (Stryer, 1981). The demonstration of the enzyme would not only signify the existence of mitochondria but also confirm the presence of citric acid cycle.

(b) Materials and methods

Whole salivary glands were obtained from 3-day fed, infective, adult H. anatolicum anatolicum and divided into two groups, A and B as described before. The method used for the demonstration of succinic dehydrogenase activity was modified after the technique described by Kerpel-Fronius and Hajos (1968). Both groups A (control) and B (for succinic dehydrogenase activity demonstration) were incubated unfixed in ferricyanide medium (Kerpel-Fronius and Hajos, 1968). While group B specimens were

incubated in a medium complete with the substrate, viz. sodium succinate (Analar grade; BDH), the control samples were incubated in a substrate-free medium. During the preparation of the medium, the solutions listed below were mixed with vigorous shaking in their numerical order: (i) 3 ml 0.5M sodium potassium tartrate (Analar grade; BDH) dissolved in Sorensen's phosphate buffer, pH 7.6, (ii) 0.35 ml 0.3M cupric sulphate (Analar grade; BDH), (iii) 0.8 ml 0.1M Sorensen's phosphate buffer, pH 7.6, (iv) 0.7 ml 0.1M sodium succinate*, and (v) 0.15 ml 0.05M potassium ferricyanide (Analar grade; BDH). The solution obtained was green and completely clear and was of pH 6.6. Samples were incubated in the medium at 37°C for 45 minutes.

The incubation was followed by 3 x 10-minute changes in Sorensen's phosphate buffer pH 7.6. They were then fixed in 4% paraformaldehyde (TAAB) in 0.1M cacodylate buffer, pH 7.4 for 30 minutes at 4°C. The fixation was followed by 4 x 30-minute changes in 0.1M cacodylate and post-fixation in 1% OsO_4 in 0.1M cacodylate buffer for 1½ hours at 4°C. After several changes in 0.1M cacodylate buffer, the tissues were dehydrated in a series of mixtures of water with component A Durcupan embedding medium, embedded and polymerised at 50°C in Durcupan/Araldite mixtures as detailed above for lactic dehydrogenase. Ultrathin sections of the polymerised tissue blocks cut with LKB glass knives on a Cambridge-Huxley Mark II Ultramicrotome were mounted on 200-mesh copper grids, stained with freshly prepared vanadatomolybdate solution as detailed previously and examined using a Philips 400 electron microscope.

*Was omitted from the medium in which the control (group A) was incubated

6.3.4 Cytochrome oxidase

(a) Introduction

The events of the electron-transport chain have been exhaustively discussed by Racker (1976), Boyer et al. (1977) and Stryer (1981). The chain comprises five cytochromes between the reduced coenzyme, QH_2, and O_2. Cytochromes b and c_1 are components of the QH_2-cytochrome c reductase complex which transfers electrons to cytochrome c. The reduced cytochrome c then transfers its electrons from the complex to the cytochrome c oxidase complex, which contains cytochromes a and a_3, the terminal members of the electron-transport chain which exists as a complex and sometimes called cytochrome oxidase. The electrons are transferred to the cytochrome a moiety of the complex, and then to cytochrome a_3, which contains copper as an electron carrier. The copper atom alternates between the oxidised, Cu^{2+}, and the reduced, Cu^{+}, states as it transfers electrons from a_3 to O_2. Without the cytochrome complex, all the components of electron-transport chain would remain in a reduced state and no ATP would be generated.

The method used in this study to demonstrate cytochrome oxidase activity was developed by Seligman, Karnovsky, Wasserkrug and Hanker (1968) and is based upon the oxidative polymerisation of 3,3'-diaminobenzidine (DAB) to a brown osmiophilic reaction product which occurs in a non-droplet form and accurately delineates the localisation of cytochrome oxidase within mitochondria. Cytochrome oxidase is specifically demonstrated via cytochrome c which, in the first step of cytochrome oxidase reaction, removes electrons from DAB (Nachlas, Crawford, Goldstein and Seligman, 1958).

The reduced cytochrome c is subsequently reoxidised by cytochrome oxidase (Cammer and Moore, 1973; Roels, 1974). The reaction product formed on oxidation of DAB by cytochrome c is further oxidised by OsO_4 vapour to an osmium black (Seligman et al., 1968).

(b) Materials and methods

Whole salivary glands, obtained by dissecting 3-day fed, adult H. anatolicum anatolicum ticks heavily infected with T. annulata, were divided into two groups, A (negative control) and B. Both groups were fixed in 4% paraformaldehyde in 0.1M sodium phosphate buffer, pH 7.4 for 45 minutes at 4°C. Fixation was followed by 3 x 20-minute changes in 0.1M sodium phosphate buffer, pH 7.4 at 4°C. After the washing, both groups A and B salivary glands were incubated in the incubation medium for 60 minutes at 37°C. The medium for the incubation of group A (negative control) contained a cytochrome oxidase inhibitor, potassium cyanide. The incubation medium as described by Seligman et al. (1968), was of the following composition: (i) 5 mg DAB (Sigma Chemical Co.), (ii) 9 ml 0.1M sodium phosphate buffer, (iii) 1 ml catalase (20 µg/ml distilled water) from bovine liver, twice crystallized suspension in water, containing 0.1% thymol; activity: 30,000-40,000 sigma units/mg protein (Sigma Chemical Co.), (iv) 10 mg cytochrome c, type VI from horse heart (Sigma Chemical Co.), (v) 750 mg sucrose. The medium finally attained pH 7.4. 6.5 mg potassium cyanide were added to the incubation medium for the negative control group A salivary glands. The incubation was followed by a wash in 0.1M sodium phosphate buffer, pH 7.4 for 20 minutes and osmication in

1% OsO_4 in 0.1M cacodylate buffer, pH 7.4 for 1½ hours at 4°C. After osmication the tissues were washed in several changes of 0.1M cacodylate buffer, dehydrated and embedded as detailed earlier for lactic dehydrogenase ultracytochemistry. Ultrathin sections of the polymerised tissue blocks, cut with LKB glass knives on a Cambridge-Huxley Mark II ultramicrotome and mounted on 200-mesh copper grids were stained with freshly prepared vanadatomolybdate solution (Callahan and Horner, 1964) as detailed before. Examination of stained sections was undertaken using a Philips 400 electron microscope.

6.3.5 Results

A strong lactic dehydrogenase activity was demonstrated by large, highly electron-dense, osmium black deposits both in the cytosol and within mitochondria (Figure 38).

Succinic dehydrogenase activity was demonstrated by the localisation of electron-dense, osmium black deposits not only on the mitochondrial membranes, but also in the contiguous matrix (Figure 39).

Cytochrome oxidase activity, demonstrated by electron-dense DAB oxidation products, was exclusively localised between the inner and outer mitochondrial membranes (Figure 40).

No reaction products were demonstrated in any of the control samples.

Figure 38 T. annulata sporozoites fixed and processed for lactic dehydrogenase activity within whole salivary glands of 3-day fed, heavily infected H. anatolicum anatolicum. A strong lactic dehydrogenase activity is demonstrated by large, highly electron-dense deposits both in the cytoplasm and within mitochondria of the parasite.
Magnification, x 127,273

Deposit arrowed

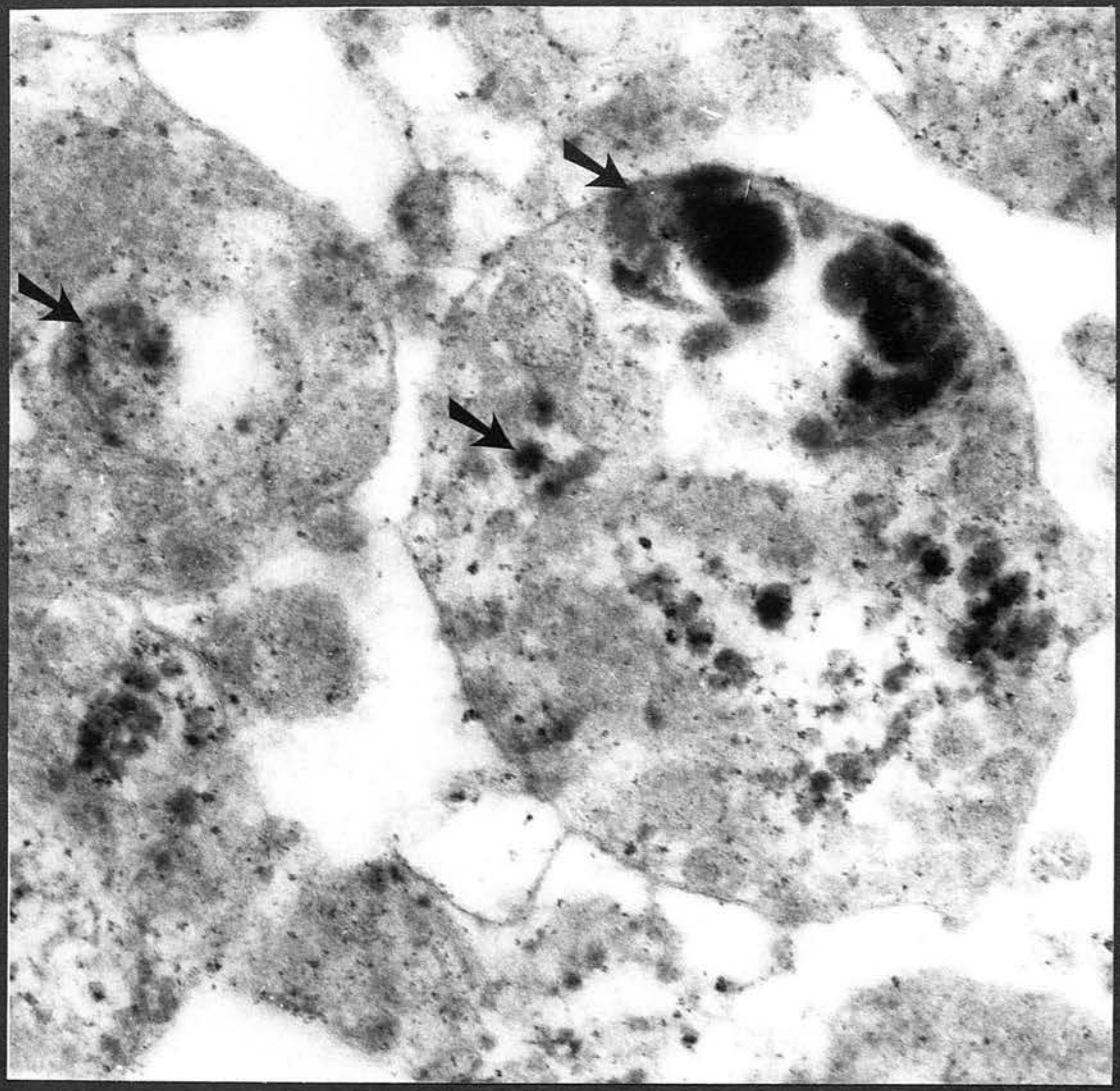
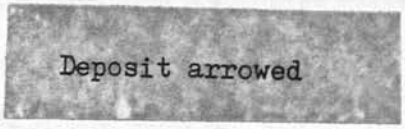


Figure 39 A T. annulata sporozoite fixed and processed for succinic dehydrogenase activity within whole salivary glands of 3-day fed, heavily infected H. anatolicum anatolicum. Succinic dehydrogenase activity is demonstrated by the localisation of electron-dense, osmium-black deposits on the mitochondrial membranes as well as in the contiguous matrix of the parasite.

Magnification, x 168,667



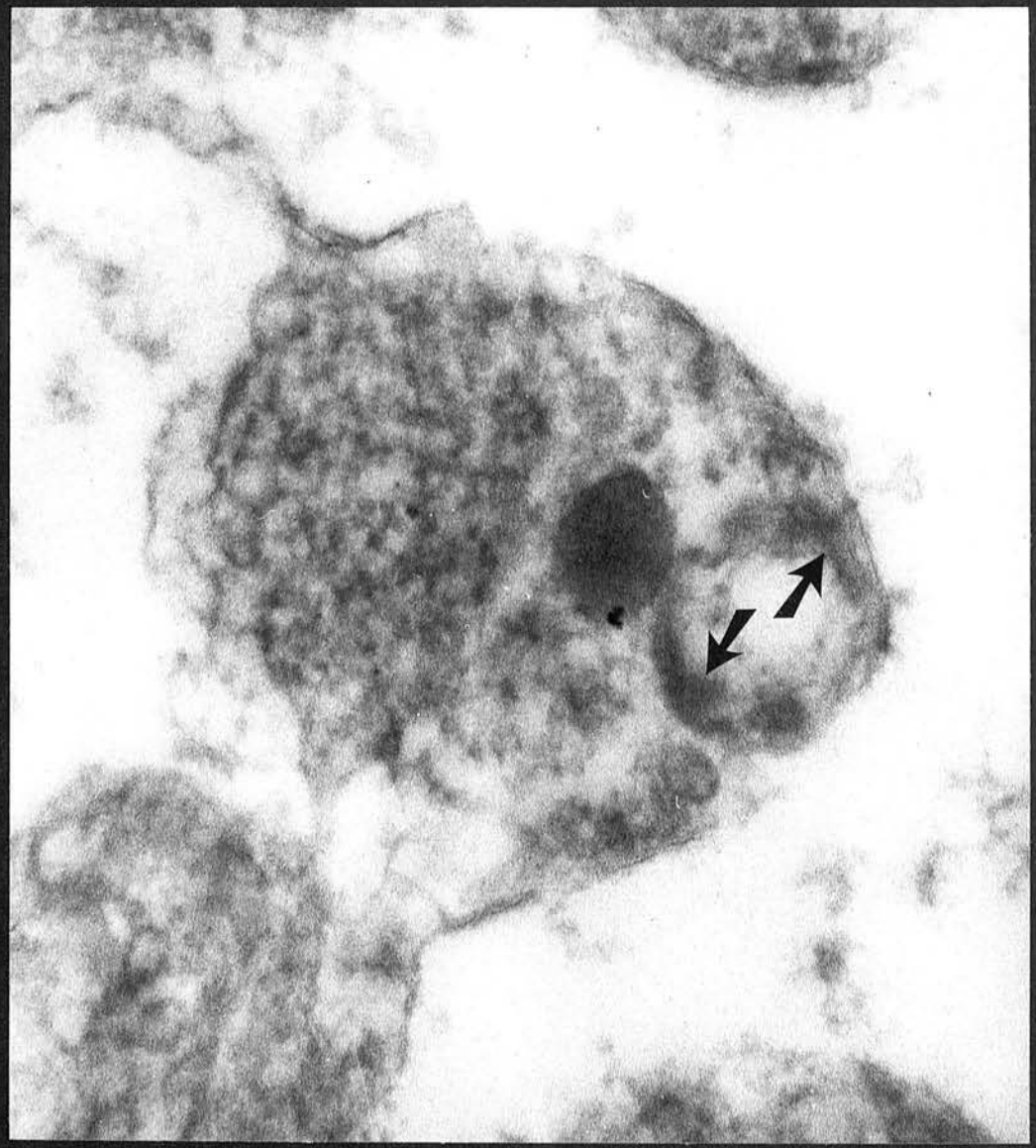
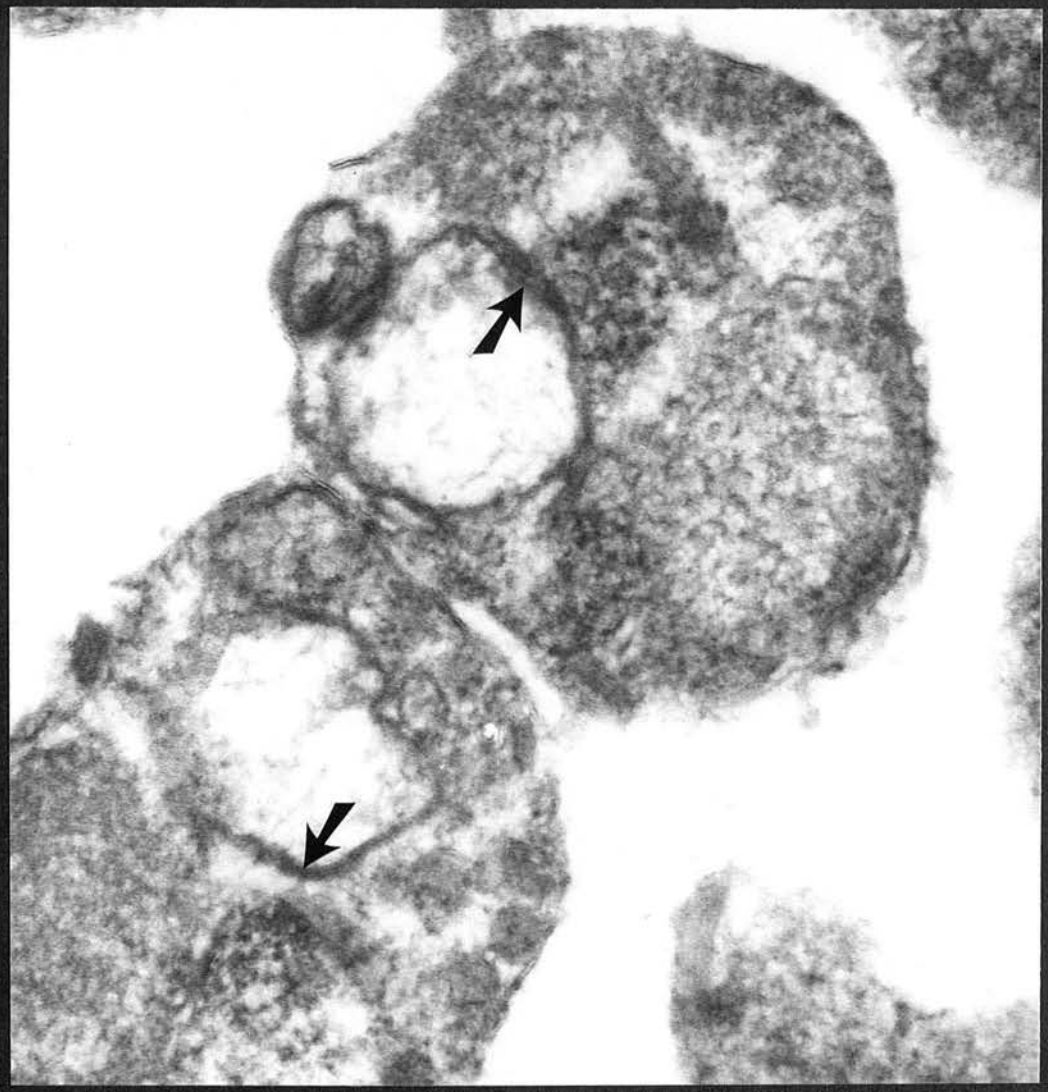


Figure 40 T. annulata sporozoites fixed and processed for cytochrome oxidase activity within whole salivary glands of 3-day fed, heavily infected H. anatolicum anatolicum. Cytochrome oxidase activity, demonstrated by electron-dense DAB oxidation products, is exclusively localised between the inner and outer mitochondrial membranes of the parasite.
Magnification, x 96,923

Deposit arrowed



6.4 Discussion

In this chapter, investigations using drugs which are known to specifically inhibit glycolysis, various steps in the electron-transport chain, and ATP synthesis (Table 14) have all shown a highly significant correlation between the dose of the drug and the number of T. annulata sporozoites invading bovine peripheral blood lymphocytes. An inverse relationship between the dose of the inhibitor drugs and the number of interiorised T. annulata sporozoites was depicted so that as the molar concentrations of the reagents increased, the number of the parasites within the lymphocytes decreased. These observations reflect a corresponding increasing inhibitory effect of the various reagents used in this study on the respective metabolic energy pathways. It would appear from this study that extracellular T. annulata sporozoites require metabolic energy for their interaction with, and invasion of, bovine peripheral blood lymphocytes, and that they possess the various metabolic energy pathways in an intact, functional state so that they independently generate the necessary ATP. That these pathways exist within the parasite in its infective stage within the salivary glands is confirmed by the ultracytochemical demonstration of the relevant marker activities (Figures 38, 39 and 40).

Like Theileria annulata, other members of the subkingdom Protozoa such as plasmodia (Mack and Vanderberg, 1978; Sherman, 1979), Babesia (Weber, 1980; 1982), Leishmania (Hart and Coombs, 1982), trypanosomes (Bayne, Muse and Roberts, 1969; Njogu, Whittaker and Hill, 1980; De Meirelles and De Souza, 1982) and Giardia lamblia (Lindmark, 1980) have been shown to possess metabolic energy pathways.

CHAPTER SEVEN

INTRACELLULAR *THEILERIA*-HOST LYMPHOCYTE INTERACTIONS IN LYMPHOBLASTOID CELL LINES TRANSFORMED BY *T. ANNULATA* AND *T. PARVA*

CHAPTER SEVEN

INTRACELLULAR *THEILERIA*-HOST LYMPHOCYTE INTERACTIONS IN LYMPHOBLASTOID CELL LINES TRANSFORMED BY *T. ANNULATA* AND *T. PARVA*

7.1 Introduction

When *Theileria* sporozoites invade target lymphoid cells both the parasite and the host cell undergo morphologic and functional changes. In Chapter 4 of this thesis, studies on the early developmental stages of *T. annulata* showed that sporozoites of this parasite dedifferentiated into trophozoites within 30 minutes of interiorisation, commenced phagotrophic feeding and growth, accompanied by the development of elaborate profiles of endoplasmic reticulum, and numerous polysomes. The trophozoites transformed into schizonts 24 hours later by an acentric closed mitosis. Observations on the interaction between *T. parva* and bovine peripheral blood lymphocytes *in vitro* based on studies by light and Nomarski interference contrast microscopy (Brown, 1979; Stagg *et al.*, 1981) and electron microscopy (Fawcett *et al.*, 1982b), have shown that the developing parasites undergo morphological changes which result in the development of schizonts. The infected host lymphoid cells undergo marked blastoid transformation and repeated replicative cycles for as long as optimal culture conditions are maintained (Malmquist *et al.*, 1970). The indeterminate number of cyclical replications and persistent state of transformation of the infected host cells is dependent on the intracellular residence of the schizont (Malmquist *et al.*, 1970) and rapidly terminates with the elimination of the parasite following

which the lymphoblast reverts to its normal morphological status (Irvin and Stagg, 1977; McHardy, 1978; Pinder et al., 1981).

In established Theileria-transformed cultures, a clone of schizont-parasitised lymphocytes is selected by recurrent bipartitioning of schizonts and distribution of daughter schizonts, with accompanying nuclei, into each daughter cell during host cell mitosis (Hulliger et al., 1964). This was demonstrated by ultrastructural studies of T. parva-transformed lymphoblasts (Vickerman and Irvin, 1981; Musisi, Bird, Brown and Smith, 1981). A mutualistic relationship is thus established between the schizont and the host lymphoid cells, the parasite itself being an obligate symbiont.

The mechanisms by which Theileria schizonts manipulate the metabolic pool of their host cells, and the regulatory and monitory devices employed by the parasites to achieve the accurate coincidence between its nuclear division and the host cell mitosis are intriguing and unknown.

In this chapter, the interactions between the schizont stage of T. annulata and T. parva and the host lymphoblasts are investigated not only ultrastructurally and by quantitating schizont nuclear numbers at various stages of host cell cycle, but also by light microscope autoradiography.

7.2 Ultrastructure

7.2.1 Materials and Methods

While PBL were separated from buffy coat obtained from defibrinated blood and layered onto a Ficoll/sodium diatrizoate gradient as detailed in Chapter 3, sporozoites of T. annulata were

prepared by grinding 3-day fed, infective adult H. anatolicum anatolicum ticks in MEM/3.5% BPA, centrifuging the suspension at 100 xg for five minutes and filtering the supernatant GUTS through a sterile 8 μm membrane filter. The filtrate was used at a concentration equivalent to 4 ticks/ml. T. parva sporozoites were prepared from 4-day fed R. appendiculatus ticks using procedures identical to those employed for preparing T. annulata GUTS as summarised above, and detailed in Chapter 3 on general materials and methods.

Both T. annulata and T. parva cultures were established in 2-cm^2 wells of tissue culture cluster plates. T. parva cultures were established in plates in which monolayer cultures of bison lung fibroblastic cell line, IMR 31, had been established 2-3 days previously to serve as feeder layers. Feeder layers were not necessary for the initiation of T. annulata cultures. 0.25 ml PBL suspension (2×10^6 cells) and 0.25 ml GUTS filtrate of either T. annulata or T. parva were seeded into each culture well. The cultures were immediately placed into a humidified plastic box, gassed with 5% CO_2/95% air and incubated at 37°C. Maintenance of the cultures was undertaken according to Brown (1979a) as detailed in Chapter 3.

Samples for electron microscopic examination were obtained at 5, 10, 20, 30, 40 and 60 minutes, 2, 3, 6, 18, 24 and 48 hours of primary cultures and during passages of established cultures. They were centrifuged at 275 xg for ten minutes at 20°C (1,450 rpm, MSE Minor). The pellets were resuspended in 5 ml serum-free RPMI 1640 (Moore et al., 1967) warmed to 37°C and spun at 275 xg for ten minutes. The resultant pellets were processed for routine electron microscopy as detailed in Chapter 3.

7.2.2 Results

For comparative purposes, representative ultrastructural characteristics of non-infected bovine peripheral blood lymphocytes are detailed in Chapter 4 of this thesis (Figure 11). Since transformed lymphoid cultures infected by either T. annulata or T. parva manifested similar host-parasite interaction features, a general description of the pertinent ultrastructural characteristics is presented.

Concomitant with the development of the interiorised Theileria parasites, the infected bovine lymphocytes were observed to undergo well-defined, progressive and marked morphological transformation. The mean cell size increased from 7 μm in the non-infected lymphocytes to 18-20 μm in the lymphoblasts transformed by Theileria parasites. There was a progressive centrifugal loosening, dispersion and eventual disappearance of chromocentres and chromonemata so that these heterochromatic regions were replaced by the more metabolically active and electron-lucent euchromatin (Figure 41). The nucleolus, which in the majority of transformed cells was intimately associated with the inner nuclear membrane (Figure 41), underwent a marked differentiation from its original interphase ring shape (Figure 11) to a reticular configuration composed of several pale centres surrounded by material of varying density and granularity (Figure 41). The host cell cytoplasm contained numerous clusters of ribosomes, the polyribosomes, 600-800Å in diameter.

The schizont stages of T. annulata (Figure 42) and T. parva (Figure 43) were observed to be tightly associated with porous,

Figure 41 An electron micrograph of a Theileria-transformed lymphoblast showing mainly the nuclear and nucleolar changes. The chromocentres and chromonemata shown in Figure 11 have loosened and converted to euchromatin while the nucleolus has undergone elaborate differentiation. Several pale centres are surrounded by dense and granular components of the nucleolus.
Magnification, x 32,124

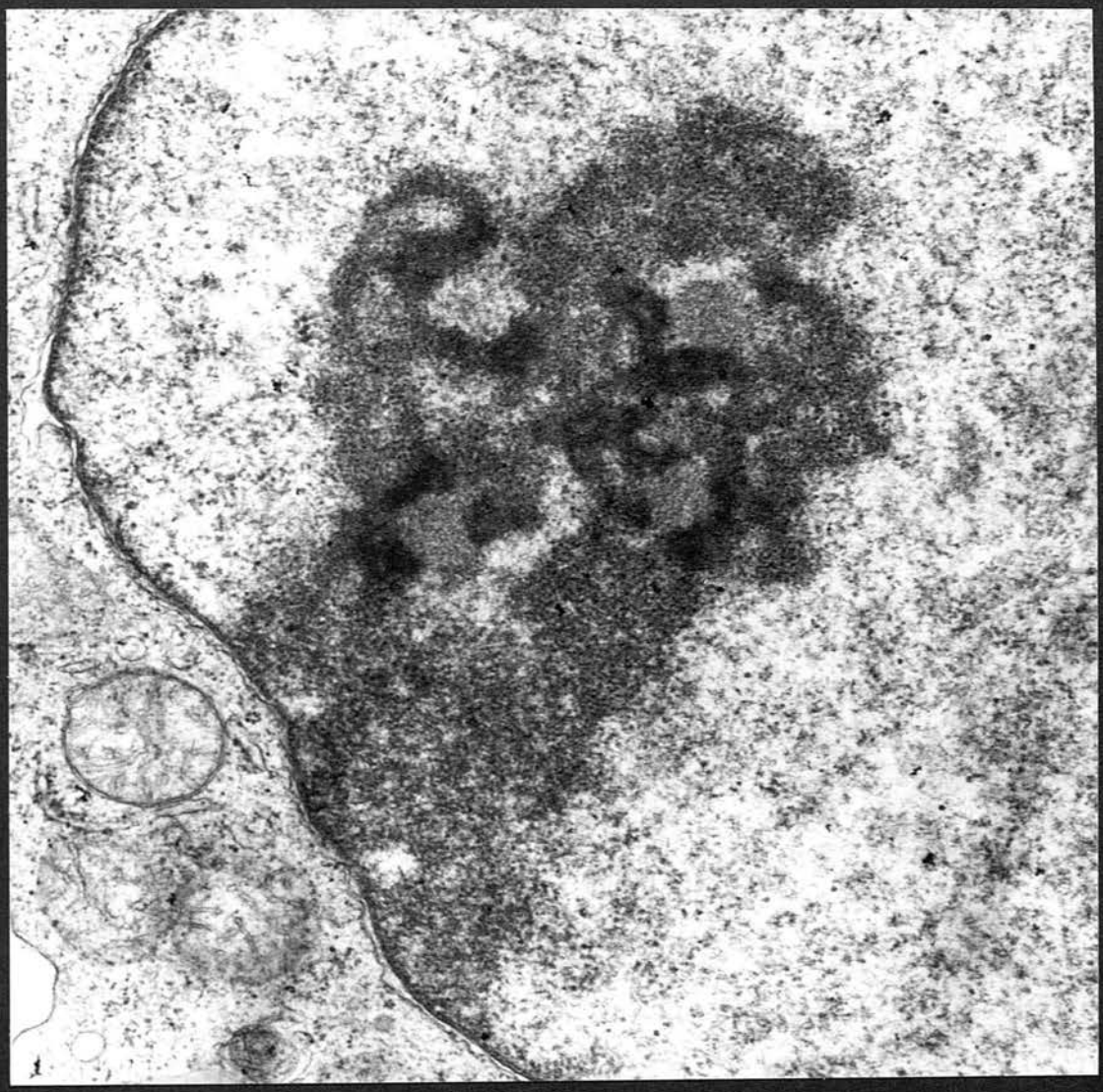
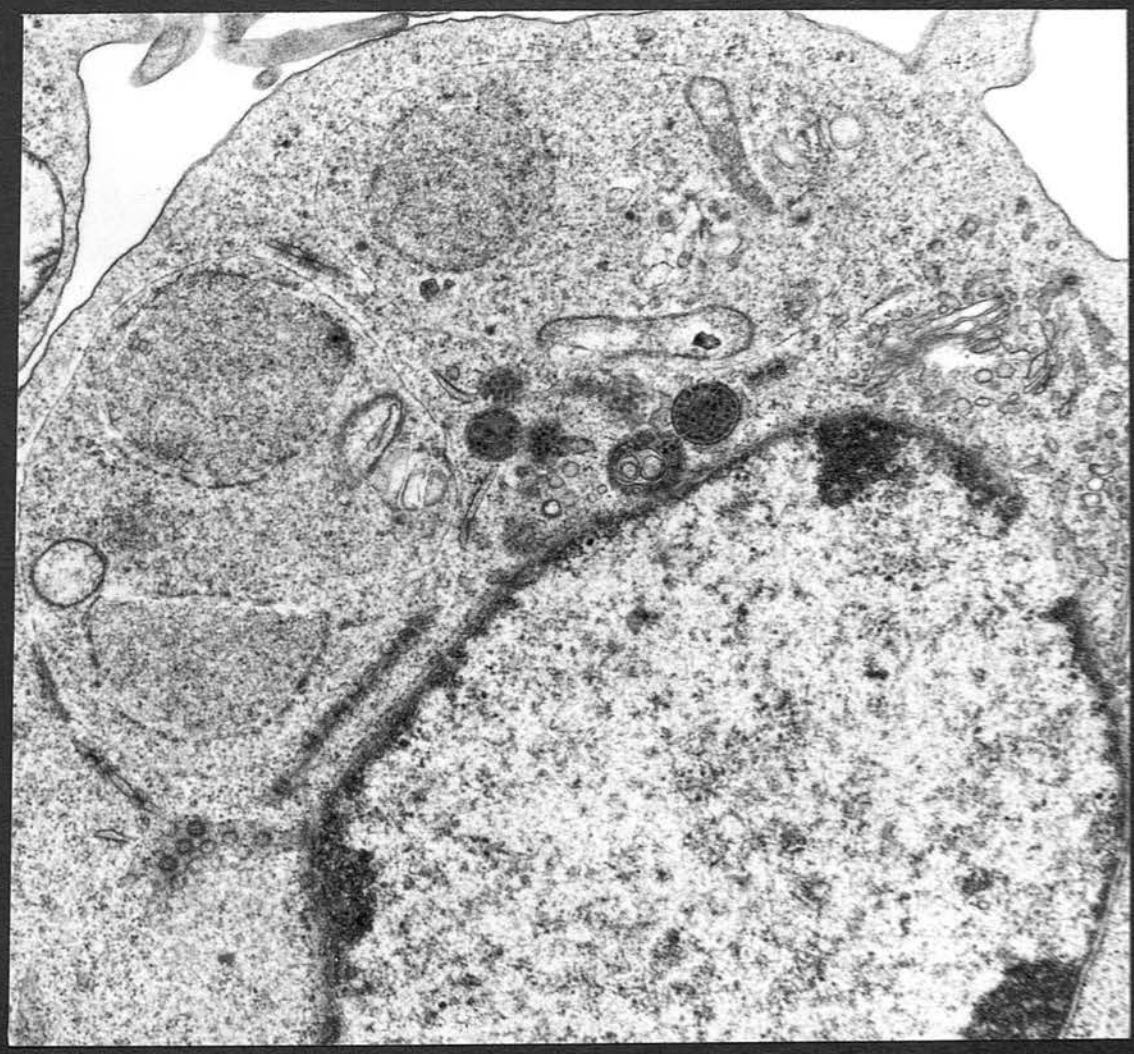


Figure 42 T. annulata schizont, shown in its host lymphocyte cytoplasm, is lying very close to the host cell nucleus. Porous annulate lamellae are shown originating from the outer nuclear membrane of the host cell nucleus by a delamination process and adopting an intimate association with the schizont. Several nuclei and mitochondria of the schizont are illustrated. Magnification, x 32,577

Figure 43 A bovine lymphocyte infected and transformed by T. parva. Two T. parva schizonts, lying adjacent to each other in the cytoplasm of the host lymphoblast, are in close proximity to the host nucleus. As shown in the case of T. annulata (Figure 42), annulate lamellae originate from the outer nuclear membrane and are tightly associated with the schizonts. Magnification, x 25,000



parallel cytomembranous type of annulate lamellae (PAL) which were structurally similar to the host lymphoblast nuclear envelope. The PAL developed from the outer nuclear membrane in both species by a delamination process and was observed juxtaposed to the cytostome of the schizont (Figure 44). Segments of the annulate lamellae were subsequently detected within phagocytic vacuoles in the cytosol of the parasite (Figure 45). The presence of PAL was transitory. At one time, the majority of the schizonts were surrounded by the organelle while at another time, this association was not observed. Schizonts were shown to be well clear of the host centriole both in the interphase cell (Figure 47) and at the onset of prophase (Figure 46). In dividing, transformed host cells in the prometaphase stage of their cycle, the schizont nuclei were consistently seen to undergo mitosis (Figures 48 and 49) characterised by a rapid development of intranuclear spindle microtubules anchored at both ends in spindle pole bodies (Figure 49). Around prometaphase (Figure 49) and later stages of mitosis, host mitotic microtubules developed and were not only associated with the lymphoblast chromosomal masses, but also inserted on the schizont pellicle.

7.3 Theileria schizont nuclear number changes during the various stages and phases of host lymphoblast cycle

7.3.1 Introduction

The observation made in Section 7.2 of this chapter that the nuclei of T. annulata and T. parva schizonts consistently divided during the prometaphase stage of their host lymphoblasts

Figure 44 An electron micrograph of a section of *Theileria annulata* schizont with one of its nuclei and mitochondria lying in the cytoplasm of the host lymphoblast. Porous annulate lamellae, contiguous to the schizont, are in close apposition to the cytostome of the parasite and are about to be phagocytosed through the organelle by the schizont. Magnification, x 115,000

Figure 45 A section of a Theileria annulata transformed bovine lymphoblast showing a schizont in close proximity to the host lymphoblast nucleus and with porous annulate lamellae tightly applied to its pellicle. Fragments of annulate lamellae phagocytosed through the cytostome as shown in Figure 44 can be seen within the phagocytic vacuoles in the schizont cytosol. The host cytoplasm is studded with polyribosomes.
Magnification, x 32,738

Figure 46 A T. annulata-transformed lymphoblast just entering prophase as shown by the budding off of a procentriole from the centriole. The schizont, at this stage of host cell cycle, is still devoid of microtubules. A rich compendium of polyribosomes fills the host lymphoblast cytoplasm.
Magnification, x 16,875

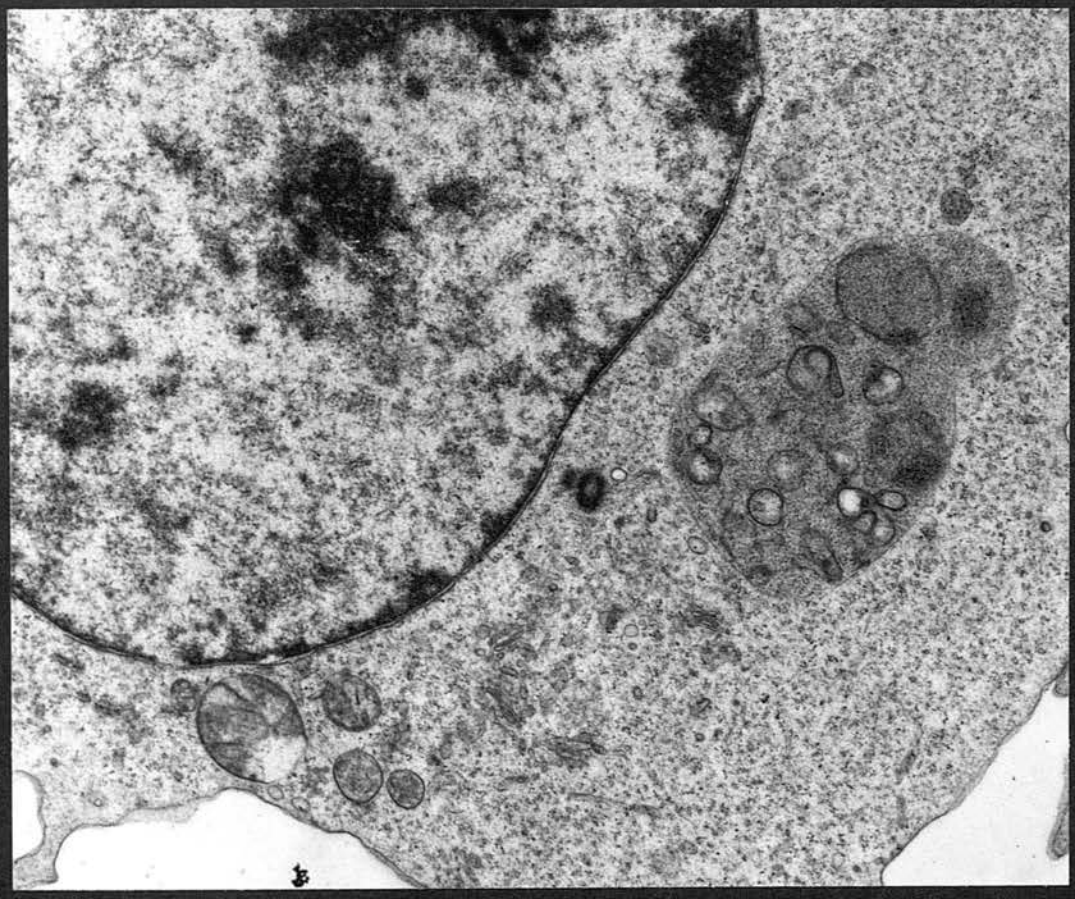


Figure 47 A section through a T. parva-transformed interphase host lymphoblast showing a part of a schizont and a centriole associated with pericentriolar satellites. The schizont is devoid of microtubules and still has no association with the host centriole together with its satellites and occasional microtubules.
Magnification, x 41,656

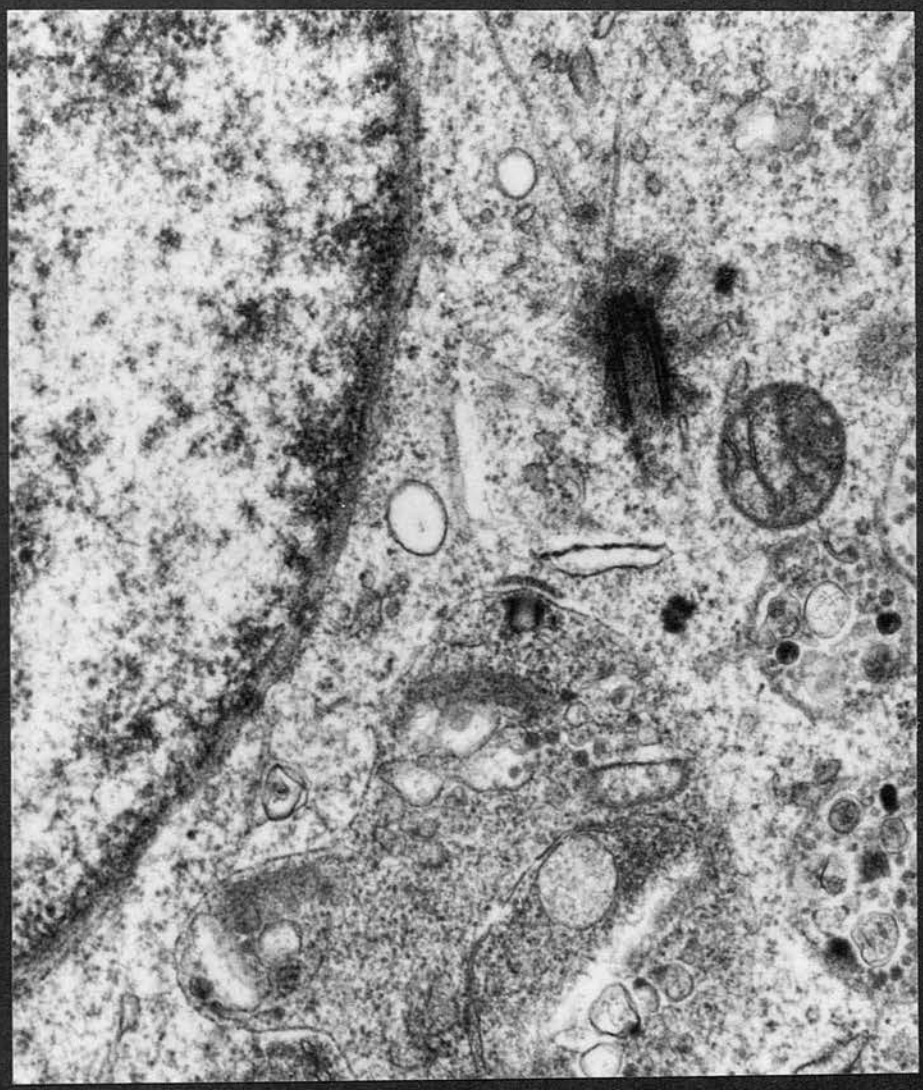


Figure 48 A T. parva-transformed lymphoblast in the pro-metaphase stage of division showing condensed chromosomal masses, host nuclear envelope fragments, well developed mitochondria and two T. parva schizonts lying with their pellicles adjacent. Several schizont nuclei are in the process of replication.
Magnification, x 26,190

Arrows depict schizonts

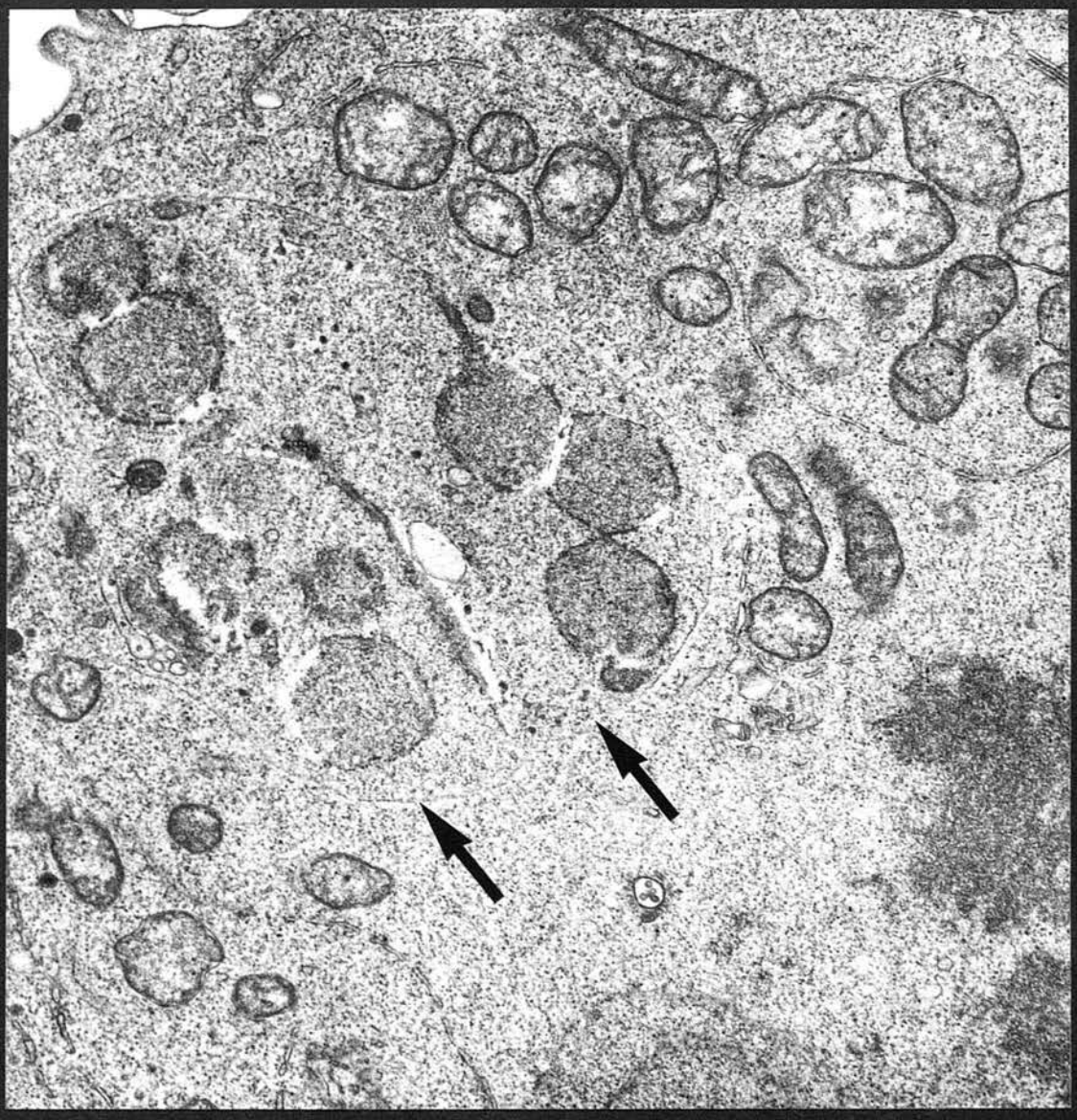
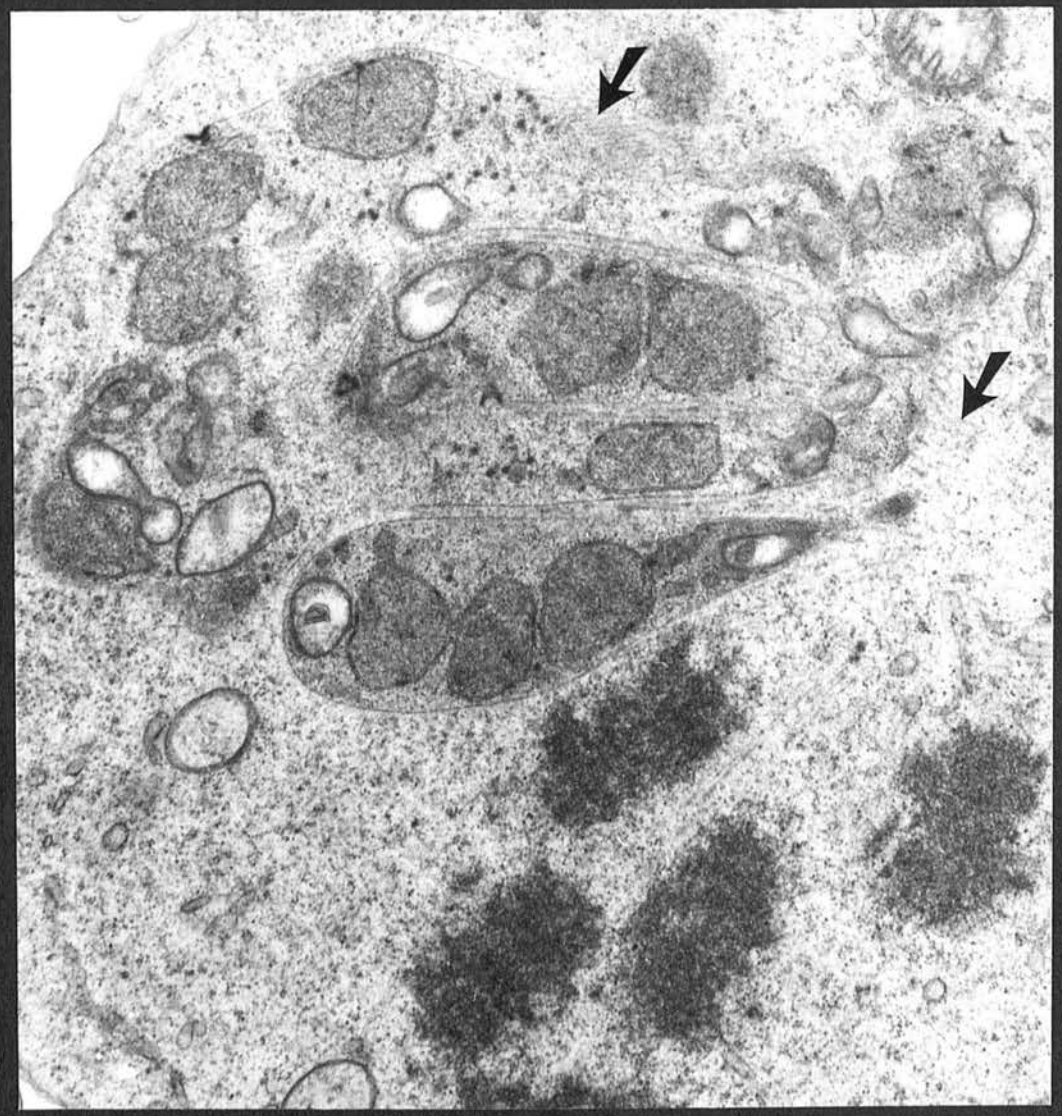


Figure 49 A T. annulata-transformed bovine lymphoblast in the late prometaphase stage showing condensed chromosomal masses and three schizonts. Several schizont nuclei are replicating. In one schizont nucleus in the early stage of replication, intranuclear spindle microtubules can be seen. Host lymphoblast spindle microtubules are beginning to develop and are embedded in the schizont pellicle and host chromosomal masses. Magnification, x 20,833

Host spindle microtubules arrowed



necessitated the quantitation of the schizont nuclear numbers of T. annulata and T. parva parasites at various stages of the host cell cycle.

In this section, such an investigation was carried out using two strains of T. annulata, viz. T. annulata (Ankara) and T. annulata (Hissar), and T. parva (Muguga).

7.3.2 Materials and methods

The three Theileria-lymphoblastoid cell lines used in this study, viz. T. annulata (Ankara), T. annulata (Hissar) and T. parva (Muguga) were subcultured at 1:4 (i.e. 1 ml of culture suspension: 4 ml fresh complete RPMI 1640) in sterile 25-cm^2 flasks 24 hours prior to sampling. This was done to ensure that the samples obtained contained culture material predominantly in the log phase of growth. During sampling, cultures were mixed thoroughly by pipetting and 50-µl aliquots obtained for cytocentrifuge smears which were air-dried, fixed in technical methanol and dried once more. Acid hydrolysis of the dried smears was carried out using a modification of the technique described by Kurtti, Munderloh, Irvin and Buscher (1981). Dried smears were transferred into staining racks containing normal hydrochloric acid, NHCl, pre-warmed to, and maintained at 60°C in a water bath, for six minutes, washed three times in Giemsa buffer, pH 7.2 and finally stained in Giemsa stain.

Schizont nuclear counts were undertaken in a total of 50, randomly selected schizont-containing lymphoblasts in each of the three cyclical categories, viz. interphase, prometaphase and anaphase-telophase in each of the three above cell lines, viz. T. annulata (Ankara), T. annulata (Hissar) and T. parva (Muguga).

7.3.3 Results

A comparison, by t-test, of the mean number of schizont nuclei of T. annulata (Ankara), T. annulata (Hissar) and T. parva (Muguga) during interphase, prometaphase and anaphase-telophase stages of host lymphoblast cycle is presented in Table 15. In all the three cell lines, the mean number of schizont nuclei at host prometaphase was highly significantly greater than the counterpart at interphase ($P < 0.001$). Similarly, the mean schizont nuclear number at lymphoblast anaphase-telophase was highly significantly greater than that at interphase ($P < 0.001$). A comparison between the prometaphase and anaphase-telophase mean schizont nuclear numbers in all the three cell lines revealed no significant difference ($P > 0.05$).

7.4 Light microscope autoradiographic study of deoxyribonucleic acid (DNA) synthesis in Theileria schizont and in the host lymphoblast

7.4.1 Introduction

In Section 7.3 of this chapter, it was observed that the mean schizont nuclear number at host lymphoblast prometaphase was more or less double the mean number at interphase in each of the cell lines investigated, viz. T. annulata (Ankara), T. annulata (Hissar) and T. parva (Muguga). In addition, it was observed that the mean schizont nuclear number at prometaphase was statistically similar to that at anaphase-telophase. These observations indicate that the division of nuclei of Theileria schizonts takes place predominantly during the prometaphase stage of the host lymphoblast

Table 15 Comparison of the mean schizont nuclear numbers of T. annulata (Ankara), T. annulata (Hissar) and T. parva (Muguga) during interphase, prometaphase and anaphase-telophase stages of the host lymphoblast cycle

Cell line	Number of observations	Interphase(1)	Prometaphase(2)	Anaphase-telophase(3)
T. annulata (A)	50	20.9 ± 11.08	35.1 ± 9.83	38.3 ± 13.65
T. annulata (H)	50	19.5 ± 9.50	39.1 ± 11.48	41.5 ± 11.87
T. parva (M)	50	20.0 ± 6.03	41.1 ± 16.18	45.1 ± 18.04

Significance of differences in the number of schizont nuclei based on equivalent t-values

	(1) Vs (2)	(1) Vs (3)	(2) Vs (3)
T. annulata (A)	$t_{(98)} = 6.777^{***}$	$t_{(98)} = 6.998^{***}$	$t_{(98)} = 1.345$
T. annulata (H)	$t_{(98)} = 9.271^{***}$	$t_{(98)} = 10.211^{***}$	$t_{(98)} = 1.036$
T. parva (M)	$t_{(98)} = 8.626^{***}$	$t_{(98)} = 9.322^{***}$	$t_{(98)} = 1.173$

***P < 0.001

cycle, well before the division of the host cell and implies that synthesis of DNA material by the schizont takes place at an earlier stage than prometaphase and not at host mitosis as suggested by Irvin et al. (1982).

In this chapter, DNA synthesis was investigated by light microscope autoradiography, in exponentially multiplying Theileria-lymphoblastoid cultures using incorporation of tritium-labelled, specific DNA precursor, thymidine (Friedkin et al., 1956), as a measure of DNA synthesis.

7.4.2 Materials and methods

The investigation was undertaken using T. annulata (Ankara), T. annulata (Hissar) and T. parva (Muguga) cell lines. Sixteen hours before pulse-labelling, cell lines were sub-cultured in a total of 5 ml complete RPMI 1640. Whereas T. parva cell line was passaged at 2 x 10^5 cells/ml, the two T. annulata cell lines were each sub-cultured at 10^5 cells/ml. Pulse-labelling of the cultures, undertaken at 16 hours following sub-culturing using 10 μCi/ml (6-^{3}H) thymidine, coating of labelled smears with Ilford nuclear L4 emulsion, developing of coated smears in D19 developer, and fixing of the developed smears in Kodak rapid fixer, were carried out as detailed in Chapter 3 of this thesis. Smears were examined for schizont and host lymphoblast nuclear labelling using either an Ortholux or Dialux 20 microscope. Four hundred schizont-containing lymphoblasts, in each smear, were examined at x 1,000 using an oil-immersion-fluotar NPL 100/1.30 objective. Smears were prepared at 16½, 20 and 24 hours. Photography was carried out using an Orthomat microscope camera (Ernst Leitz).

7.4.3 Results

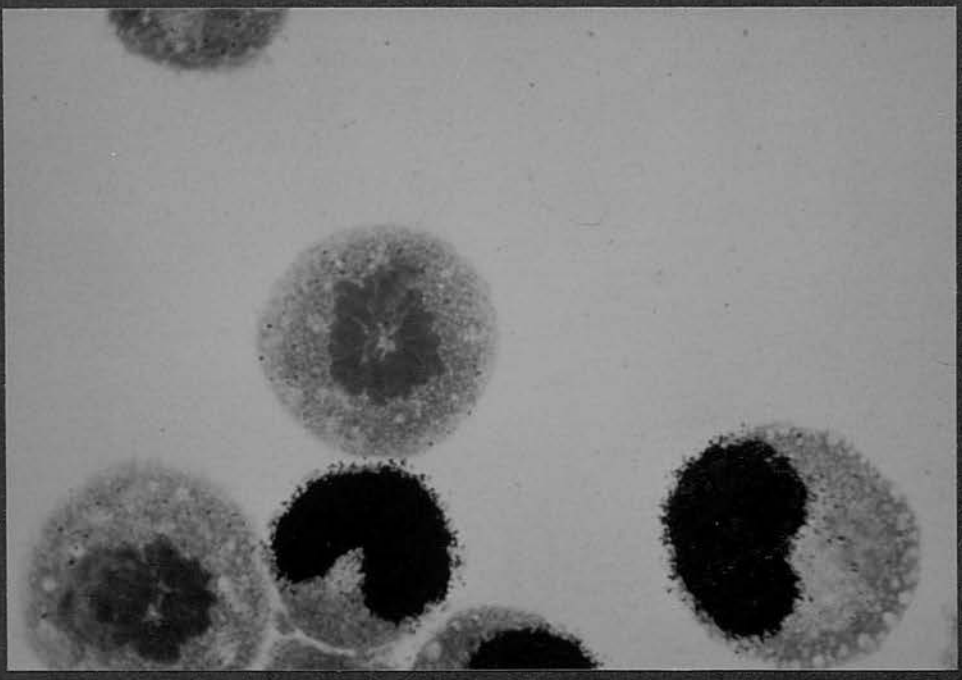
The pattern of incorporation of tritiated thymidine was similar in both T. annulata and T. parva cell lines. In smears prepared immediately after pulsing (16½ hours) the percentage of interphase lymphoblast nuclei labelled with tritiated thymidine in T. annulata and T. parva cell lines were 48.3 and 65.1 respectively. In such labelled lymphoblasts, as well as in mitotic host cells whose nuclei did not incorporate tritiated thymidine, schizont nuclei were not labelled (Figure 50). At the same time, immediately after pulse-labelling, labelled schizont nuclei were demonstrated only in non-labelled interphase host lymphoblasts (Figures 51 and 52). In smears prepared at four hours and eight hours after pulse-labelling of the cultures, labelled schizonts were demonstrated in non-labelled mitotic cells (Figures 53, 54 and 55) in both T. annulata and T. parva cell lines. In the same smears labelled mitotic host lymphoblasts (Figure 56) appeared. Although in smears prepared immediately after pulsing, no mitotic cells contained labelled schizonts, four hours later, as many as 47.3 and 57.8% of mitotic cells, non-labelled mitotic lymphoblasts transformed by T. annulata and T. parva respectively, contained labelled schizonts.

7.5 Discussion

In this study it has been demonstrated that infection of bovine peripheral blood lymphoid cells with Theileria sporozoites results in the manifestation of the panoply of characteristic ultrastructural features that define the transformed lymphoblastoid state. The chromocentres and chromonemata, composed of inactive, tightly

Figure 50 A Giemsa-stained, cytocentrifuge smear of Theileria annulata transformed lymphoblasts labelled with tritiated thymidine and processed for light microscope autoradiography. The smear, prepared immediately after a 30-minute pulse, demonstrates that no schizont nuclei incorporated tritiated thymidine in either mitotic host cells or in host lymphoblasts in the S-phase whose nuclei were labelled.
Magnification, x 1,280

Figure 51 A Giemsa-stained, cytocentrifuge smear of Theileria annulata transformed lymphoblasts processed for light microscope autoradiography as in Figure 50. The smear, prepared immediately after a 30-minute pulse-labelling, shows two label-negative interphase cells, one with prominent schizont nuclear labelling and the other negative for schizont nuclear labelling.
Magnification, x 1,600



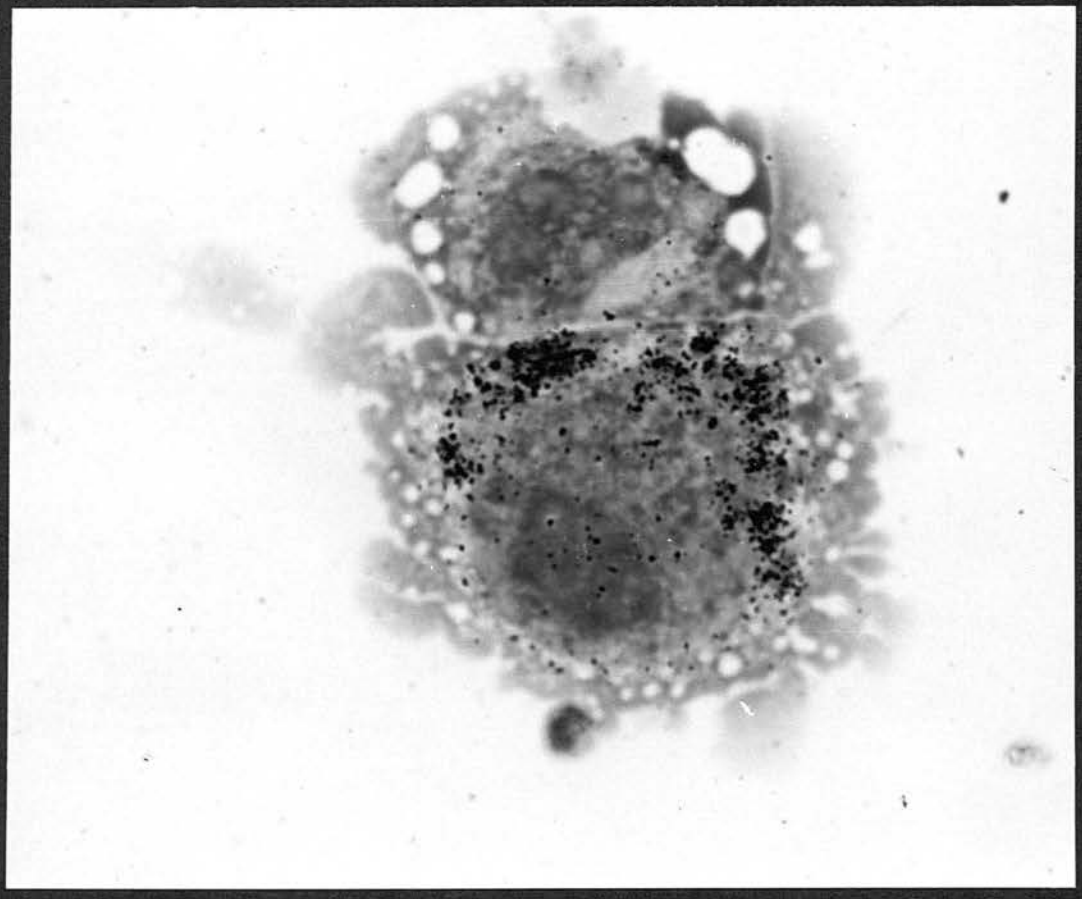


Figure 52 A light microscope autoradiograph of Theileria parva transformed lymphoblasts. The smear was prepared immediately following a 30-minute pulse and demonstrates incorporation of tritiated thymidine by schizont nuclei in label-negative interphase cells. The S-phase interphase cells incorporating tritiated thymidine are negative for schizont nuclear labelling.
Magnification, x 1,600

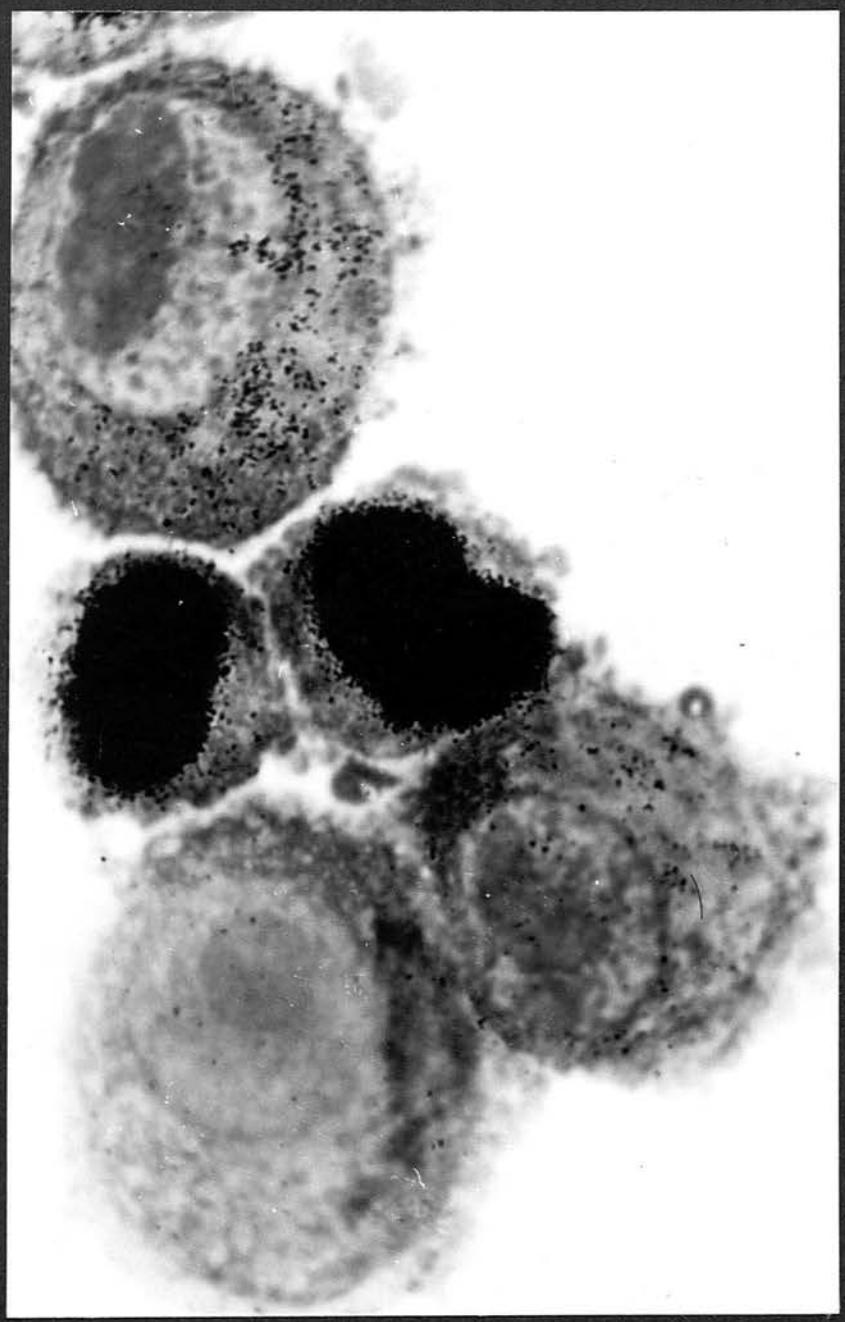
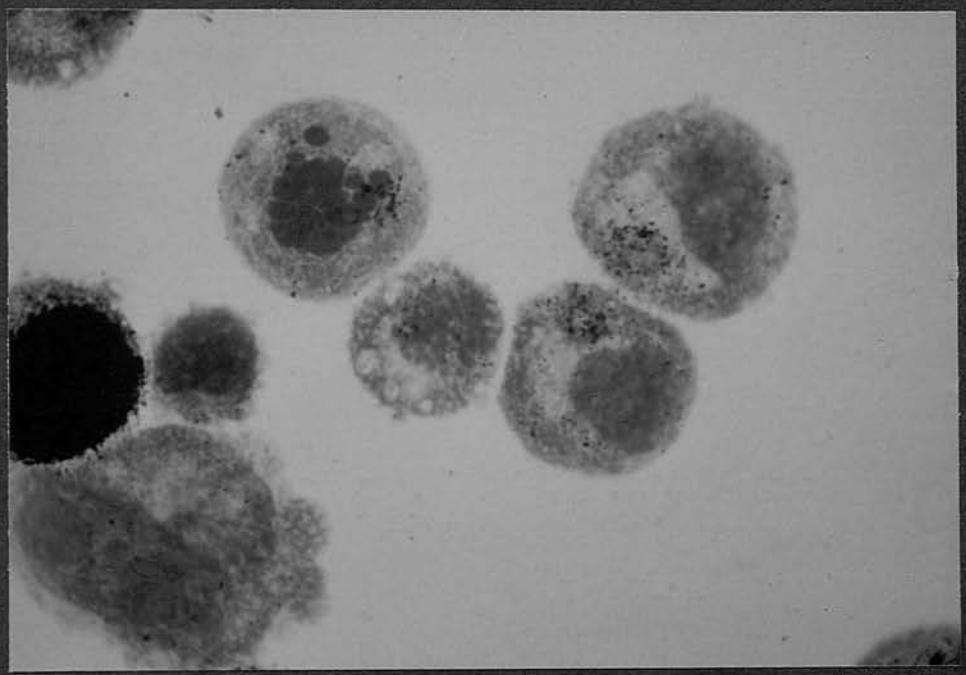


Figure 53 A light microscope autoradiograph of Theileria annulata transformed lymphoblasts. The smear, prepared at 4 hours and 8 hours following pulse-labelling shows labelled schizont nuclei within label-negative mitotic host cells.
Magnification, x 1,280

Figure 54 A light microscope autoradiograph showing two Theileria parva transformed lymphoblasts in a smear prepared 4 hours following pulse-labelling. While the S-phase interphase cell in the smear is negative for schizont nuclear labelling, the label-negative mitotic cell demonstrates labelled schizont nuclei distributed among host chromosomal masses, ready for distribution into daughter lymphoblasts.
Magnification, x 1,600



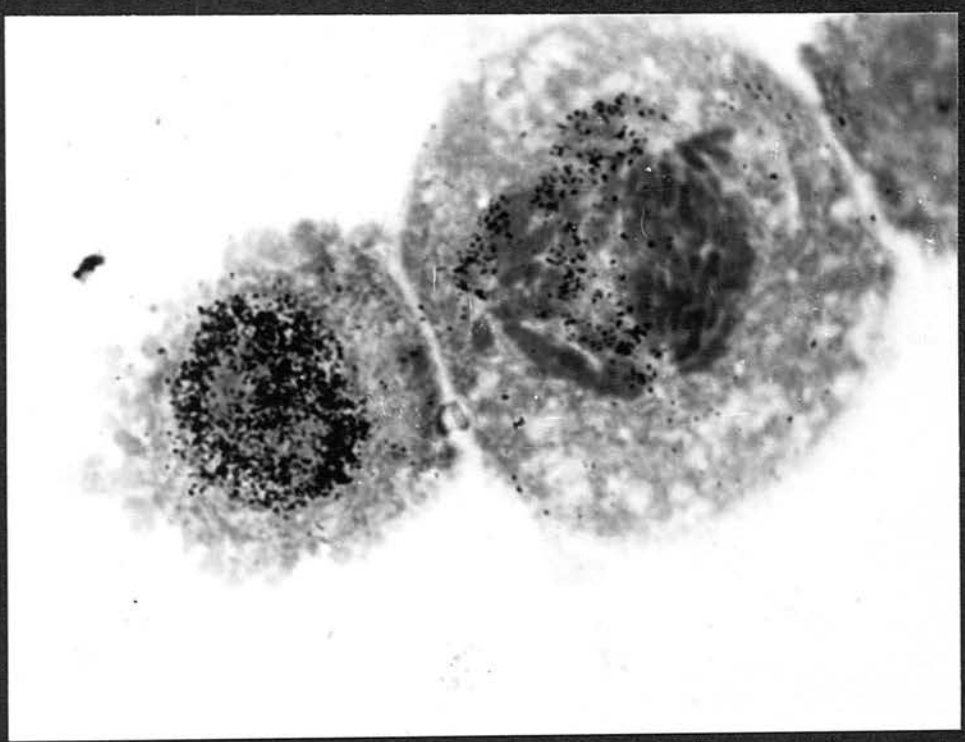


Figure 55 An autoradiograph of a Theileria parva transformed, mitotic lymphoblast in a smear prepared 4 hours following pulsing. The schizont nuclei have incorporated numerous grains of tritiated thymidine while the host cell is label-negative. Magnification, x 1,600

Host cell chromosomal masses (CHM)

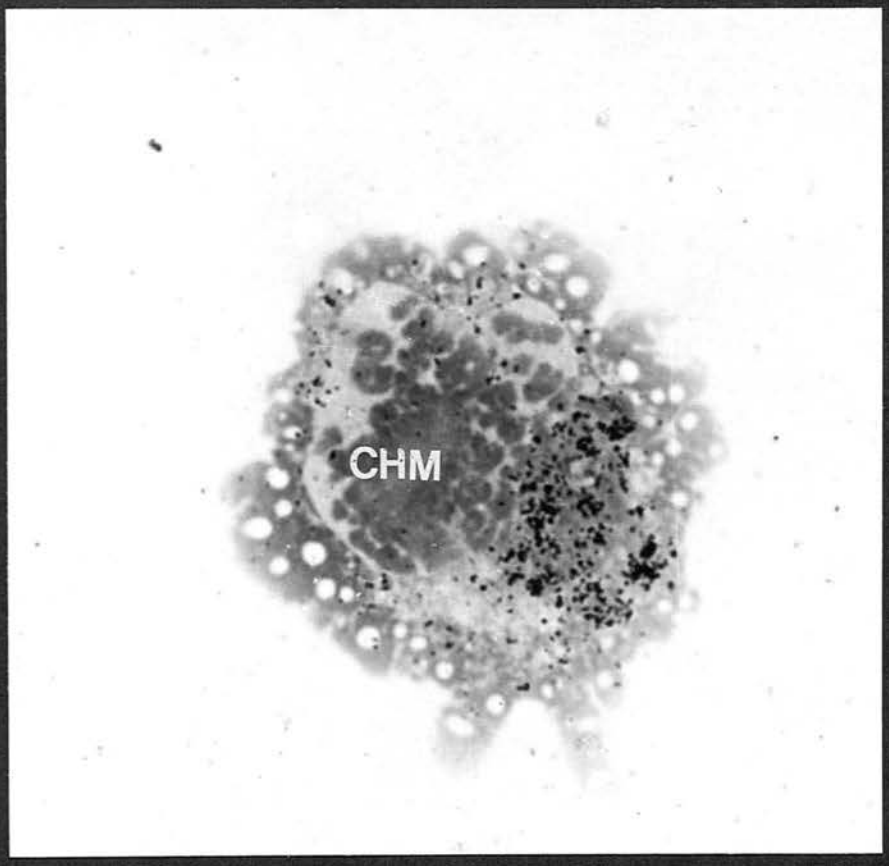
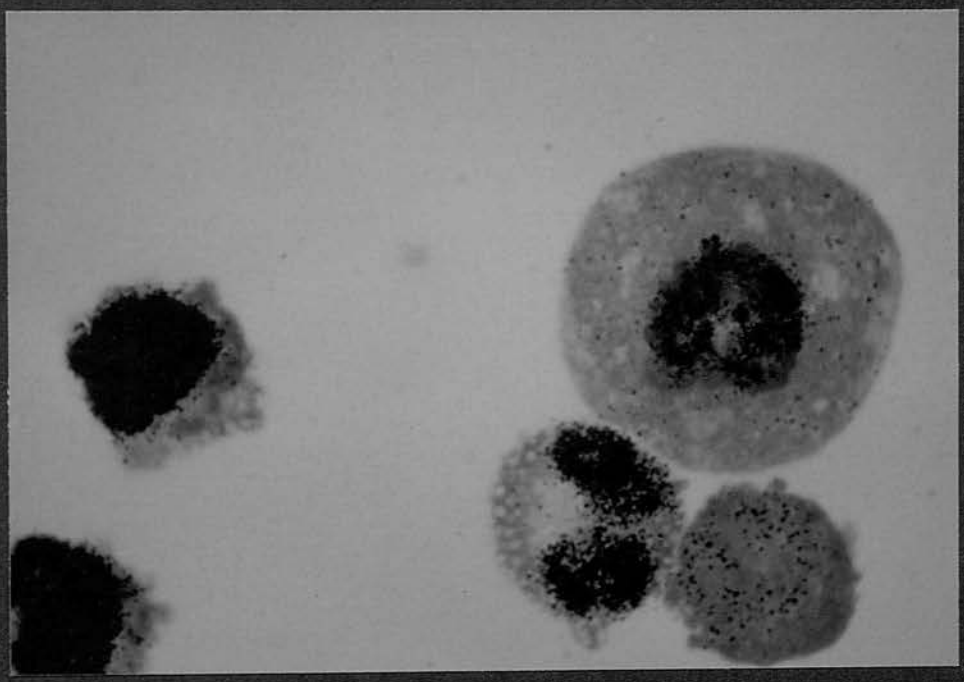

CHM

Figure 56 An autoradiograph of Theileria annulata transformed lymphoblasts in a smear prepared 4 hours following pulse-labelling. No schizont labelling is seen in either S-phase, labelled interphase cells or labelled mitotic lymphoblasts. Magnification, x 1,280



wound and concentrated chromatin (Figure 11), gradually loosen, disperse and convert to euchromatin, characteristic of nuclei of Theileria-transformed lymphoblasts (Figure 41). Autoradiographic studies on thymocyte nuclei using Uridine-H^3 (Allfrey and Mirsky, 1962; Sibatani, de Kloet, Allfrey and Mirsky, 1962; Littau, Allfrey, Frenster and Mirsky, 1964) and Uridine-2-C^{14} (Frenster, Allfrey and Mirsky, 1963) demonstrated the ribonucleic acid (RNA) precursors are incorporated within euchromatic regions and that DNA in these regions actively synthesise RNA which is largely messenger ribonucleic acid (mRNA). The amount of euchromatin in a nucleus reflects the number of genes being transcribed rather than the amount of mRNA produced (Hopkins, 1978). The diffuse distribution of euchromatin observed in the nuclei of Theileria-transformed lymphoblasts would thus implicate the multiplicity of genes participating in the transcription of new mRNAs, and a correspondingly large number of heterogeneous translation products.

Another characteristic structural transformation involves the nucleolus which differentiates into a large reticular structure (Figure 41) in which the original amorphous, pale centre subdivides into several parts and the perinuclear heterochromatin changes to an elaborate meshwork of varying density and granularity. The DNA within the nucleolar area contains multiple copies of genes involved in the production and distribution of RNA and is the site of ribosomal ribonucleic acid (rRNA) transcription (Perry, 1969; Threadgold, 1976). It has been shown by electron microscope autoradiography that transformed lymphocytes readily incorporate uridine-H^3 in nucleolar structure (Winter and Yoffey, 1966; Biberfeld, 1971).

The incorporated uridine-H^3 localises on the elaborate meshwork of dense granular material (Vagner-Capodano, Bouteille, Stahl and Lizzitzky, 1982), an observation which is compatible with the idea that the dense granular components contain newly synthesised RNA (Busch and Smetana, 1970). The extensive differentiation and prominence of the nucleolus in Theileria-transformed lymphoblasts, therefore, signifies a marked increase in nucleolar activity and turnover of rRNA, implying enhanced protein synthesis.

Concurrent with the nuclear and nucleolar changes described in this study is the development of a rich compendium of polyribosomes in the cytoplasm of host lymphoblasts. In vivo studies using C^{14}-labelled amino acids with subsequent lysis of cells (Warner, Rich and Hall, 1962; De Man and Noorduyn, 1969) have demonstrated that the site of protein synthesis is not the single ribosome, but rather the multi-ribosome-mRNA complexes, the polyribosomes, since it is these complexes which are capable of incorporating C^{14}-labelled amino acids into protein. In the complexes, the ribosomes act as the co-ordinating unit relating mRNA and tRNA while the strand of mRNA which holds the ribosomes together contains the information for assembling the tRNA-amino acid couple to the polypeptide chain. There is, therefore, no doubt from the above discussion that the infection of lymphoid cells with theilerial parasites induces a complex, interrelated chain of reactions at molecular level that underly the characteristic lymphoblastoid or transformed state.

Annulate lamellae (Swift, 1956) have been most commonly described in rapidly differentiating cells such as oocytes, spermatocytes,

embryonic as well as tumour and cancer cells (Kessel, 1968; 1973; Wischnitzer, 1970; Franke, 1974; Maul, 1977). Their occurrence, but not their origin, in Theileria lymphoblast cultures has been documented (Musisi, Bird, Brown and Smith, 1981; Vickerman and Irvin, 1981). Direct evidence that they originate from the nuclear envelope was first provided in studies on the developing Necturus oocyte (Kessel, 1963) and subsequently verified in developing echinoderm oocytes (Kessel, 1964) and tunicate oocytes (Kessel, 1965). Material associated with annulate lamellae in the dragon-fly oocyte (Kessel and Beams, 1969), Drosophila melanogaster spermatocytes (Kessel, 1981) and sea urchin eggs (Conway, 1971) has been shown by cytochemical methods to contain RNA. Although numerous studies have been undertaken on annulate lamellae, their functional significance remains speculative. It has been suggested that they: (1) carry nuclear-derived information to the cytoplasm (Moses, 1964; Swift, 1958); (2) are involved in the assembly of stored gene products and activation of ribosomal subunit material into functional polyribosomes (Kessel, 1981); and (3) contribute towards the formation of cytoplasmic microtubule complex (Chemnitz and Salmberg, 1978; Musisi, Bird, Brown and Smith, 1981). It has been observed that annulate lamellae appear not only transitorily or cyclically (Wischnitzer, 1970; Maul, 1977), but also that they are present at specific times. For example, in maturing human spermatids, annulate lamellae are observed at stages designated Sb1 and Sd1 (Smith and Berlin, 1977), while in developing chick myocardial cells, they are present only on the 11th day and not on the 7th or 8th day of incubation (Merkow and Leighton, 1966). The observations that annulate lamellae originate from the nuclear

envelope, are associated with RNA and appear at specific stages in developmental events imply that they contain coded instructions necessary either for initiating, regulating or monitoring certain cellular activities.

The demonstration in this investigation that Theileria schizont nuclei consistently divided at host cell prometaphase (Figures 48 and 49), as corroborated by the observation that the mean schizont nuclear number at host prometaphase-(anaphase-telophase) is more or less double the mean nuclear number at interphase (Table 15), depicts the ability of the schizont to stimulate lymphoblastoid transformation, and to monitor host cyclical activities and precisely time its own nuclear replication. In preparation for this nuclear multiplication, the schizont synthesises its DNA material. It has been shown in this study that schizont nuclear DNA synthesis, as determined by incorporation of tritiated thymidine in smears prepared immediately after a short pulsing, took place in interphase lymphoblasts whose nuclei were not labelled (Figures 51 and 52) and not in mitotic cells as previously reported by Irvin et al. (1982). In this study, mitotic cells examined immediately after a short pulsing of cultures were devoid of labelled schizont nuclei (Figure 50). It is possible that Irvin et al. (1982) prepared the first set of smears much later than 30 minutes after pulsing as a result of delayed washing off of the excess tritiated thymydine. Four hours following pulsing, however, labelled schizont nuclei were demonstrated in non-labelled mitotic lymphoblasts (Figures 53, 54 and 55) which formed 47.3 and 57.8% of mitotic cells in T. annulata and T. parva cell lines respectively. These

label-negative mitotic cells with labelled schizonts must have derived from the label-negative interphase host lymphoblasts which contained labelled schizonts in smears obtained immediately after pulsing. This implies that the label-negative interphase cells with labelled schizonts must have synthesised their DNA before the pulsing was carried out and were, therefore, in the G2 phase of the interphase stage at the time of pulse-labelling, merely waiting to enter prophase and proceed to mitosis.

The nuclear, nucleolar and polyribosomal changes described in this study, coupled especially with the host nuclear-annulate lamellar-schizont interactions together with the precise timing of schizont nuclear DNA synthesis and replication are of considerable interest and provoke some very challenging questions about the molecular mechanisms underlying Theileria-induced host lymphoid cell transformation, and the functional significance of annulate lamellae in Theileria-transformed lymphoid cell lines.

It is suggested that Theileria parasites, consequent on interiorisation, provoke the blastoid transformation and the formation of annulate lamellae through the influence of components of their genomic material on host cell DNA and that the annulate lamellae are a transcription product representing a species of mRNA and serve as a monitoring device for the schizont, communicating cyclical changes in host cell nucleus to Theileria schizonts so that through their degradation and assimilation (Figure 45), the schizont is able to follow the host lymphoblast chromosomal changes and replicate its nuclei in the prometaphase stage as demonstrated in this study (Figures 48 and 49). The nuclear replication of the

schizont is timed such that when host cell anaphase chromosomal migration starts, there is concurrent distribution of schizont nuclei into each daughter cell in a similar manner as for host chromosomes through the instrument of the host mitotic spindles (Figure 49). Where host spindle microtubule activity is interfered with, for example, by treatment with colchicine (Hulliger et al., 1964; Hulliger, 1965) or where the host mitotic process is disturbed by ionising radiation (Irvin, Brown and Stagg, 1975) or by actinomycin D (Moulton, Krauss and Malmquist, 1971), the schizont assesses the host nuclear activity correctly and replicates its nuclei so that up to 100 schizont nuclei may be found per cell. Whereas colchicine impairs microtubule polymerisation (Borisy and Taylor, 1976) and cells are arrested in the metaphase stage, X-irradiation of cultures blocks cells in the G2 phase from entering into mitosis due to chromosomal breakage (Das, 1963) and increases the mitotic index even up to 80% (Padilla, Van Dreal and Anderson, 1966). The schizont is more resistant than the host cell to both treatments and develops normally while the host cell replication is severely affected.

The transfer of Theileria genomic material from the lymphoblast cytoplasm to the nucleus may be achieved, as in the case of steroid hormones (Green, 1980), by complexing with specific receptor proteins in the target cell cytoplasm thus increasing their affinity for chromatin and hence the migration to the nucleus where they would recognise and bind to specific gene sites to initiate the transcription of mRNA molecules. The transcriptional events may be

spread over a number of phases so that during the first 24-48 hours of infection, for example, the majority of mRNA species produced code for trophozoite-host lymphocyte interactions. The second phase species would be responsible for the stable, perpetual and symbiotic co-existence characteristic of Theileria-lymphoblast cultures in vitro, while in vivo, the late transcriptional products may be concerned with the terminal stages of the schizont, and the accurately timed switch to piroplasm production (Jarrett et al., 1969; Radley et al., 1974; Jura and Losos, 1980). The incorporated Theileria DNA components thus represent a self-contained transcriptional unit which utilises host replicative machinery yet remains subject to influences originating from outwith itself.

The insinuation of parasite genome into host cell nucleus has also been postulated in "Xenoma" production by the microsporidian, Glugea weissenbergi in the stickleback Apeltes quadracus (Sprague and Vernick, 1968). In this case the infective sporoplasms of Glugea are believed to be introduced directly into the host cell nucleus where, as a result of the interaction between the parasite and the host cell DNAs, symbiotic complexes of multinucleate host cell and intracellular parasites, the "Xenoma" (Weissenberg, 1968), are formed. A similar relationship has been described in the turbot Scophthalmus maximus (Ferguson and Roberts, 1976; Kirmse, 1978) in which every cell of a proliferative, lymphoma-like condition was parasitised by schizonts of a protozoan parasite, Haemogregarina sachai (Coccidia; Adeleidea). Although the intermediate host for H. sachai is not yet documented (Kirmse, 1978a), its developmental stages in Scophthalmus maximus are similar to

those of Theileria parasites in their vertebrate hosts. It is possible that H. sachai may employ fractions of its genetic material, in a manner similar to that suggested in this study for Theileria parasites, to stimulate the characteristic yellowish nodular lesions of schizont-containing lymphomatous proliferations.

CHAPTER EIGHT

INTRACELLULAR *THEILERIA*-HOST LYMPHOCYTE INTERACTIONS IN BOVINE LYMPHOSARCOMA (BL-20) CELL LINE SUPERINFECTED BY THE HISSAR STRAIN OF *T. ANNULATA*, BL-20-*T.a.*(H), AND THE ANKARA STRAIN OF *T. ANNULATA* (BL-20-*T.a.*(A))

CHAPTER EIGHT

INTRACELLULAR *THEILERIA*-HOST LYMPHOCYTE INTERACTIONS IN BOVINE LYMPHOSARCOMA (BL-20) CELL LINE SUPERINFECTED BY THE HISSAR STRAIN OF *T. ANNULATA*, BL-20-*T.a.*(H), AND THE ANKARA STRAIN OF *T. ANNULATA* (BL-20-*T.a.*(A))

8.1 Introduction

BL-20 is a continuous bovine lymphoblastoid cell line established by culture (Morzaria, Roeder, Roberts, Chasey and Drew, in press) from a bronchial lymph node of a four-month old calf suffering from sporadic bovine leukosis in its multicentric form. According to the above authors, the cell line has neither surface immunoglobulin nor receptors for peanut agglutinin. In an ultrastructural study, they showed that the majority of the cells possessed, in addition to the nuclear pockets often containing cytoplasmic inclusions, heterochromatic clumps in the nucleoplasm and prominent Golgi complex associated with annulate lamellae. Karyotypically BL-20 cells were female and comprised a normal bovine chromosome complement. The cell line has a population doubling time of 24 hours. The BL-20 cell line was sent by Dr. S.P. Morzaria to the CTVM where it was infected with *T. annulata* (Hissar) and *T. annulata* (Ankara) sporozoites giving rise to the persistently-infected cell lines BL-20-*T.a.*(H) and BL-20-*T.a.*(A) and maintained according to the techniques described by Brown (1979a).

In Chapter 7 of this thesis, observations were made which led to the conclusion that blastoid transformation of normal target host lymphoid cells and obligate symbiotic co-existence *in vitro* were achieved by *Theileria* parasites through a complex, interrelated

chain of reactions at molecular level induced as a result of the insinuation of parasite genetic factors on host cell DNA. The influence Theileria infection would have on an already transformed, cancerous cell line, such as bovine lymphosarcoma, has not been investigated so far. In this chapter, ultrastructural characteristics of the host-parasite relationship and growth rates of BL-20, BL-20-T.a.(H) and BL-20-T.a.(A) were investigated. The growth rates of the latter two cell lines were also compared with those of their bovine peripheral blood lymphocyte (PBL) counterparts, viz. PBL-T.a.(H) and PBL-T.a.(A).

8.2 Ultrastructural characteristics of BL-20, BL-20-T.a.(H) and BL-20-T.a.(A)

8.2.1 Materials and methods

The BL-20 cell line (Morzaria et al., in press) was isolated from a bronchial lymph node of a four-month old calf suffering from sporadic bovine leukosis as detailed in Chapter 3 on general materials and methods. The cell line was sent to the CTVM by Dr. S.P. Morzaria in primary growth medium (PGM) which comprised Hank's buffered salt solution enriched with yeastolate (0.01%), lactalbumin hydrolysate (0.035%), foetal calf serum (20%) and antibiotics. Routine maintenance of the cell line at the CTVM was undertaken in complete RPMI 1640, passaging every three days at a ratio of 1:4 (i.e. 1 ml of BL-20 cell suspension:4 ml complete RPMI 1640 pre-warmed to 37°C) in fresh 25-cm^2, gassing with 5% CO_2/ 95% air and incubating at 37°C.

Its infection with the Hissar and Ankara strain of T. annulata and subsequent maintenance as BL-20-T.a.(H) and BL-20-T.a.(A)

respectively were undertaken at the CTVM. GUTS filtrate containing sporozoites of the Hissar and the Ankara strains of T. annulata were prepared as described in Chapter 3. The 8 μm membrane filtrate of sporozoites was used at a concentration equivalent to 4 ticks/ml of MEM/3.5% BPA.

BL-20-T.a.(H) and BL-20-T.a.(A) cell lines were set up by mixing BL-20 cell suspension with GUTS suspension of the appropriate strain of T. annulata using the method described by Brown (1979a) and detailed in Chapter 3. For electron microscopy, established lymphoblastoid BL-20, BL-20-T.a.(H) and BL-20-T.a.(A) cell lines were sub-cultured at 1:4 in 25-cm^2 plastic flasks, gassed with 5% CO_2/95% air and incubated at 37°C for approximately 24 hours.

During the exponential phase, cultures were mixed by pipetting and samples for electron microscopy obtained. Suspensions of cells from each cell line were centrifuged at 275 xg for ten minutes at 20°C (1,450 rpm, MSE Minor) and the pellets resuspended in serum-free RPMI 1640 warmed to 37°C. The samples were centrifuged again at 275 xg for ten minutes and the resultant pellets processed for routine electron microscopy as detailed in Chapter 3.

8.2.2 Results

The most frequently observed unique ultrastructural feature of BL-20 cell line was nuclear pockets (Figures 57, 58 and 59). The persistence of heterochromatic clumps in the nucleoplasm of BL-20 cells (Figure 59) was also manifest. In BL-20-T.a.(H) and BL-20-T.a.(A) cells, the conversion of heterochromatin to euchromatin was more complete, the nucleolar transformation was markedly

Figure 58 A section of a bovine lymphosarcoma cell showing two nuclear pockets.
Magnification, x 28,846

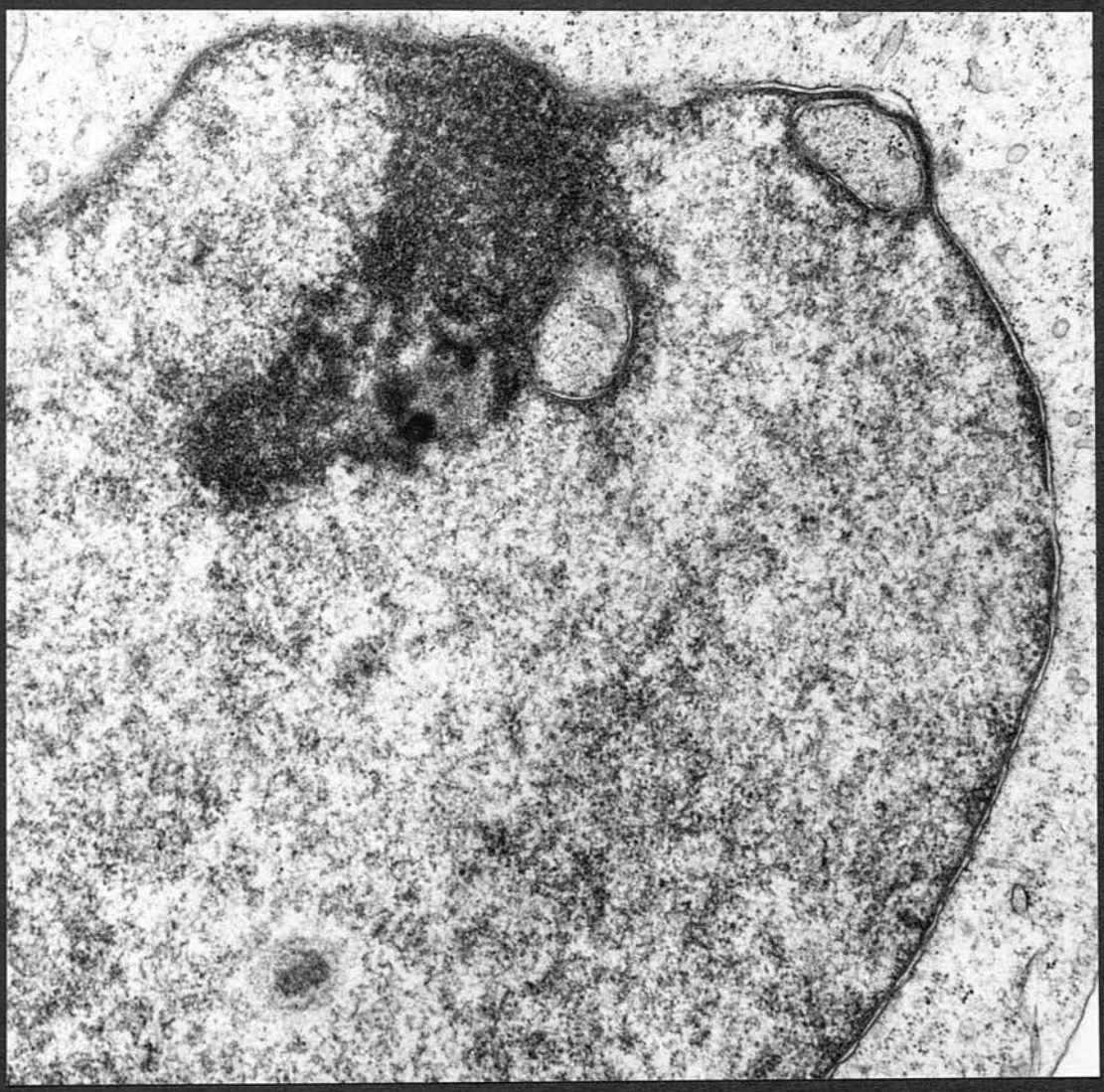
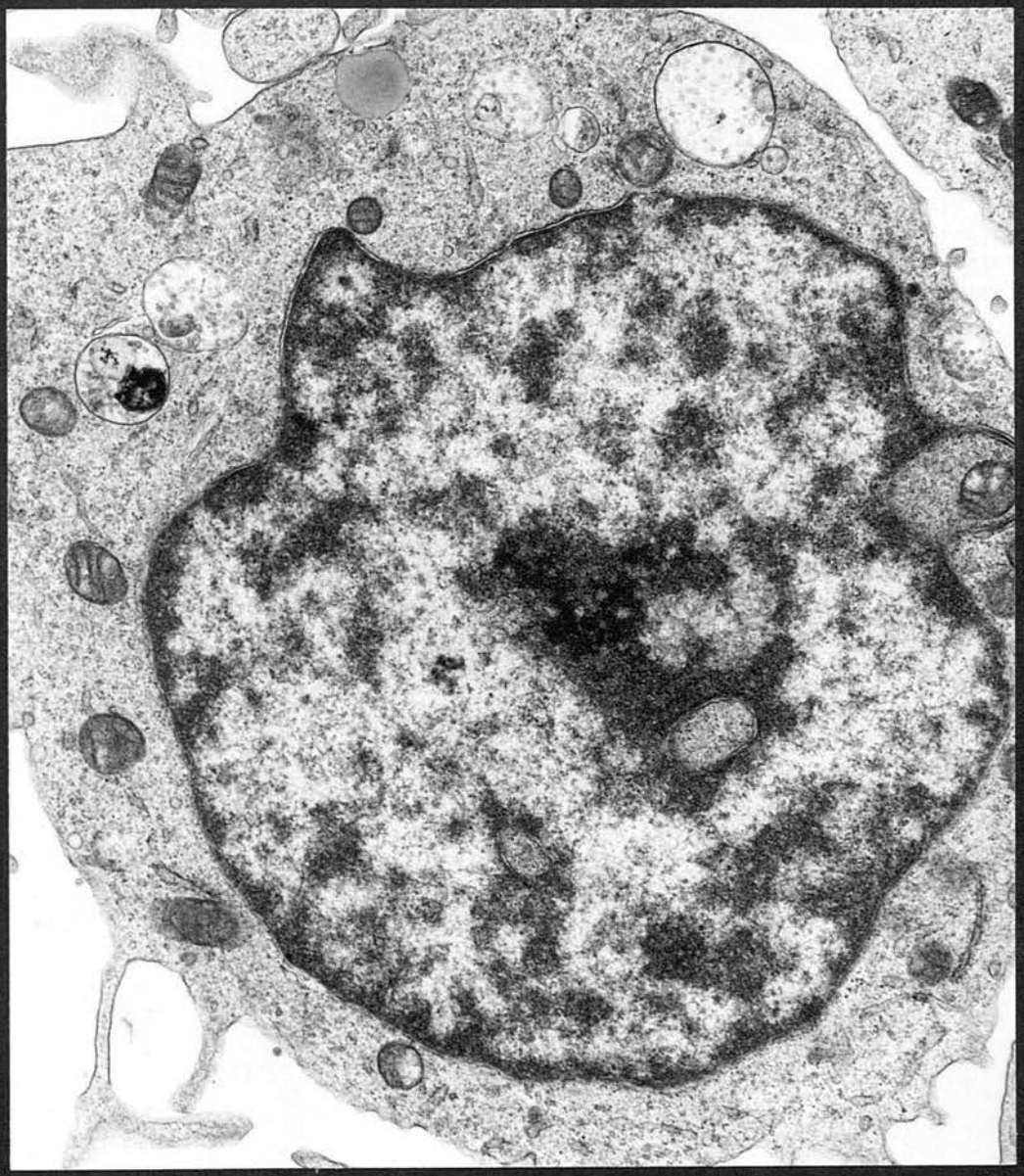


Figure 59 A bovine lymphosarcoma cell, with several nuclear pockets and numerous chromocentres, is shown.
Magnification, x 21,875



elaborate, and the intimate annulate-lamellar-schizont association was observed (Figure 60). In over 100 of the Theileria-infected BL-20 cells, no nuclear pockets were observed. Reminiscent schizont-host mitotic microtubule association in newly formed daughter lymphoblasts (Figure 60) was also occasionally observed. In BL-20-T.a.(H) and BL-20-T.a.(A) cells in late prometaphase, schizont nuclei were shown replicating, and the developing host mitotic spindle microtubules were demonstrated inserting on Theileria annulata schizont pellicle (Figure 61).

8.3 Comparison of growth rates of BL-20, BL-20-T.a.(H), BL-20-T.a.(A), PBL-T.a.(H) and PBL-T.a.(A)

8.3.1 Introduction

In PBL transformed to lymphoblasts either by T. parva (Figure 48) or T. annulata (Figure 49), schizont nuclear replication was synchronised with host lymphoblast prometaphase. In this chapter, a similar synchrony was observed (Figure 61) in lymphosarcoma cells superinfected either by T. annulata (Hissar), BL-20-T.a.(H), or T. annulata (Ankara), BL-20-T.a.(A). These observations suggest that the growth rate of cells containing Theileria schizonts is determined by the parasite to suit its own replicative rate. This implies that any cell lines infected and transformed by a particular Theileria parasite would have a similar growth rate.

In this section, a study is undertaken to compare the growth rates of BL-20, BL-20-T.a.(H), BL-20-T.a.(A), PBL-T.a.(H) and PBL-T.a.(A) cell lines.

Figure 60 An electron micrograph of a T. annulata-infected BL-20 cell showing an intimate annulate-lamellar-schizont association and elaborate nucleolar transformation. Reminiscent schizont-host mitotic microtubule association can be seen indicating that the parasite is in a recently formed daughter cell.
Magnification, x 24,096

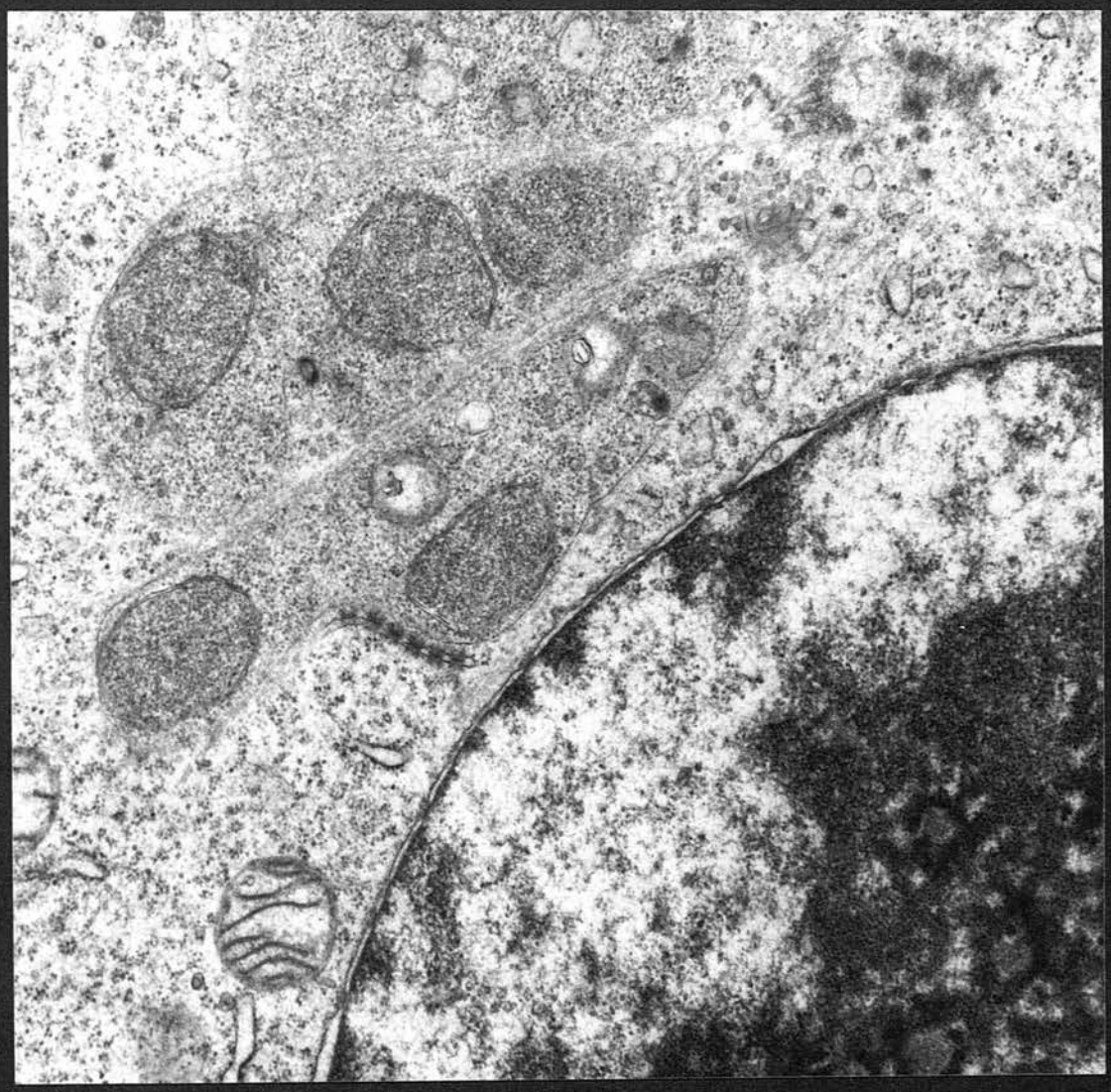
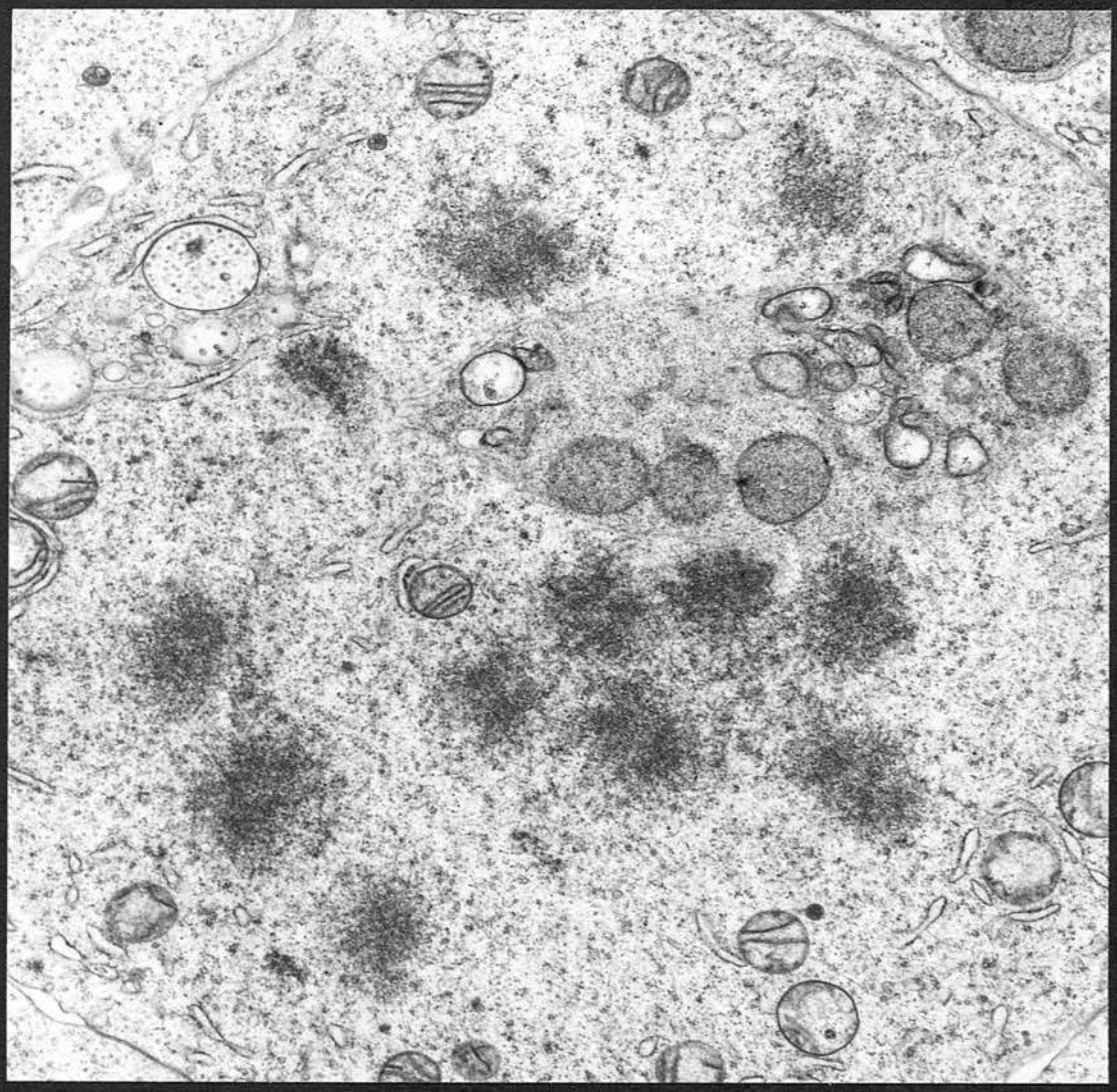


Figure 61 A T. annulata-infected BL-20 cell in the pro-metaphase stage of division. A T. annulata schizont, with some of its nuclei replicating, is seen in between the host cell chromosomal masses. Host spindle microtubules, just forming, can be seen embedding in the schizont pellicle at opposite ends.
Magnification, x 14,792.



8.3.2 Materials and methods

The viability of cells was determined using trypan blue dissolved in 0.9% NaCl (Boyum, 1968) and used as a 0.2% solution. BL-20, BL-20-T.a.(H), BL-20-T.a.(A), PBL-T.a.(H) and PBL-T.a.(A) cell lines which had been maintained in culture for more than ten passages were thoroughly mixed by gentle pipetting (except PBL-T.a.(A) which needed a more vigorous mixing since it grows mainly as a monolayer). Equal parts of each cell line and the 0.2% trypan blue solution were mixed and allowed to stand for five minutes, then counted in a New Improved Neubauer haemocytometer according to Schalm, Jain and Carroll (1975) as described before. Each cell line was then diluted with complete RPMI 1640 and seeded at 2×10^5 viable cells/ml in 5 ml complete RPMI 1640 in fresh 25-cm^2 flasks, gassed with 5% CO_2/95% air and incubated at 37°C.

On Day 1 all the cell lines were mixed by pipetting and 0.5-ml aliquots obtained for counting as described above. The cultures were immediately gassed with 5% CO_2/95% air and incubated at 37°C. On Day 2, after mixing of cultures and obtaining 0.5-ml aliquot samples for dilution with 0.2% trypan blue and subsequent counting, cell suspension from each cell line was aseptically pipetted into a sterile plastic universal bottle and centrifuged at 400 xg for five minutes at 20°C (1,800 rpm, MSE Chilspin). The pellets were resuspended in 5 ml, fresh complete RPMI 1640 pre-warmed to 37°C and returned to their original flasks. On Day 3, cultures were mixed by pipetting following which 0.5-ml aliquot samples were obtained for counting using 0.2% trypan blue, a haemocytometer, and an Ortholox microscope as described above.

8.3.3 Results

While the results are summarised in Table 16, detailed data are presented in Appendix Table 5. In all the cell lines, viz. BL-20, BL-20-T.a.(H), BL-20-T.a.(A), PBL-T.a.(H) and PBL-T.a.(A), regression analysis revealed a curvilinear relationship between cell concentration and time. The curvilinearity persisted even when semi-log transformation was used. To enable valid comparisons to be undertaken, lines of best fit of the means of the daily cell concentrations expressed as logarithms were calculated and a significant linear relationship was depicted in each cell line (Table 16). A comparison of the slopes of the regression lines by t-test (Table 16) not only revealed a significant difference ($P < 0.05$) between BL-20 and BL-20-T.a.(H), but also showed a highly significant difference ($P < 0.01$) between BL-20 and BL-20-T.a.(A). No significant differences were revealed between BL-20-T.a.(H) and BL-20-T.a.(A), PBL-T.a.(H) and PBL T.a.(A), and BL-20-T.a.(H) and PBL-T.a.(H). A significant difference was, however, revealed between BL-20 T.a.(A) and PBL-T.a.(A).

8.4 Discussion

In this chapter, it has been demonstrated that the occurrence of nuclear pockets is a characteristic feature of the lymphosarcomatous cell line, BL-20 (Figures 57, 58 and 59). Although a rare pocket may be found in some tumours, large numbers of nuclear pockets are characteristic of lymphomas and leukemias, particularly follicular lymphoma, so that their occurrence is of some diagnostic value in distinguishing lymphomas and leukemias from other neoplasms

Table 16 Regressions of log means of the daily cell concentrations and comparisons of slopes of regression lines by t-test

Cell line	Regression equation	Correlation coefficient
BL-20	$\hat{y} = 5.276 + 0.216.x$	+0.995**
BL-20-T.a.(H)	$\hat{y} = 5.272 + 0.367.x$	+0.994**
BL-20-T.a.(A)	$\hat{y} = 5.318 + 0.373.x$	+0.996**
PBL-T.a.(H)	$\hat{y} = 5.379 + 0.304.x$	+0.969*
PBL-T.a.(A)	$\hat{y} = 5.310 + 0.290.x$	+0.999***

Comparisons of slopes of regression lines

BL-20 V BL-20-T.a.(H)	$t_{(4)} = 4.522$*	
BL-20 V BL-20-T.a.(A)	$t_{(4)} = 5.815$**	
BL-20-T.a.(H) V BL-20-T.a.(A)	$t_{(4)} = 0.164$	N.S.
PBL-T.a.(H) V PBL-T.a.(A)	$t_{(4)} = 0.255$	N.S.
PBL-T.a.(H) V BL-20-T.a.(H)	$t_{(4)} = 1.018$	N.S.
PBL-T.a.(A) V BL-20-T.a.(A)	$t_{(4)} = 3.572$*	

N.S. = Not significant

*P < 0.050

**P < 0.010

***P < 0.001

(Ghadially, 1980). A strong correlation between the occurrence of nuclear pockets and chromosomal abnormalities, either unusual numbers, breakage or a defect in nucleoprotein synthesis was reported by Clausen and Von Haam (1969) who also suggested that the pockets may be a morphologic expression of chromatin dislocation consequent on such chromosomal abnormalities. A close correlation between the occurrence of aneuploidy and a high frequency of nuclear pockets has been reported not only in cases of bovine lymphosarcoma (Hare, Tsu-ju Yang and McFeely, 1967), but also in human acute leukemia (Ahearn, Trujillo, Cork, Fowler and Hart, 1974). Although this correlation was especially manifest during the active phase of the disease, successful response to therapy was accompanied by a marked reduction or total disappearance of the chromosomally and ultrastructurally abnormal cells (Ahearn et al., 1974). Conversely, imminent relapse was heralded by the reappearance of both aneuploidy and nuclear pockets (Ahearn et al., 1974).

It has been demonstrated in this study that when the BL-20 cell line is infected and transformed by Theileria annulata parasites, the nuclear pockets disappear and the host cell-parasite interactions are identical to those manifest in normal bovine peripheral blood lymphoid cells transformed to lymphoblastoid cell lines by Theileria parasites. Nuclear heterochromatin to euchromatin transformation and nucleolar differentiation (Figures 41 and 60), schizont-annulate lamellar association (Figures 42, 43 and 60), host prometaphase-schizont nuclear replication synchrony (Figures 48, 49 and 61) and host microtubule-schizont interactions in late prometaphase

(Figures 49 and 61) were common to Theileria-transformed PBL and BL-20-T.a.(H) and BL-20-T.a.(A).

The disappearance of nuclear pockets in BL-20-T.a.(H) and BL-20-T.a.(A) observed in this study simulates a similar observation in lymphosarcoma cells subjected to therapy (Ahearn et al., 1974) and implies loss of activity of the factors responsible for the cancerous nature of lymphosarcoma cells. In the context of this investigation, it suggests that Theileria takes over the host cell chromosome sites of operation and exerts regulatory activity so that resultant changes suit its metabolism and replication. This is corroborated in Table 16 where a comparison of the slopes of the regression lines for BL-20, BL-20-T.a.(H), BL-20-T.a.(A), PBL-T.a.(H) and PBL-T.a.(A) depicts a similar growth rate for Theileria transformed cell lines (except PBL-T.a.(A) V BL-20-T.a.(A) which was significantly different from that of BL-20. The significant difference observed in this study between PBL-T.a.(A) and BL-20-T.a.(A) is attributable to the fact that PBL-T.a.(A) cell line grows predominantly as a monolayer and, even with vigorous pipetting, a fair number of cells remain stuck to the bottom of the flask and are not included in the counts.

The observations made in this chapter strongly support the proposal in Chapter 7 that Theileria parasites achieve blastoid transformation of host lymphoid cells and obligate symbiotic coexistence through the influence of their genetic factors on host cell DNA.

CHAPTER NINE

GENERAL DISCUSSION

An *in situ* ultrastructural study of *T. annulata* sporozoites in whole salivary glands obtained from 3-day fed, heavily infected *H. anatolicum anatolicum* ticks, undertaken primarily to define the morphologic characteristics of mature, infective sporozoites, provided some information on sporogony. The original ramifying parasite mass appeared to give rise to smaller units which were devoid of rhoptries, had one or more nuclei and varying numbers of mitochondria, and from which multitudes of sporozoites formed by schizogony. Two mechanisms have been proposed for sporogony in *Theileria* parasites. Light microscopic studies of *T. annulata* in *H. anatolicum excavatum* (Schein and Friedhoff, 1978), *T. parva* in *R. appendiculatus* (Martin *et al.*, 1964) and *T. taurotragi* in *R. appendiculatus* (Young, Grootenhuis, Leitch and Schein, 1980) described the presence, within infected salivary gland acini, of an initial coherent parasite mass, the primary sporoblast, whose nuclei replicated and which later divided to form several secondary sporoblasts. Continued nuclear replication followed by less frequent cytoplasmic division of the secondary sporoblast gave rise to multinucleate tertiary sporoblasts or cytomeres. Sporozoites formed from cytomeres by a budding process. Fawcett *et al.* (1982a), consequent on their electron microscopic study of sporogony of *T. parva* in *R. appendiculatus*, disputed the above traditional description of sporogonic development of *Theileria*. They instead proposed that the parasite develops as a ramifying, multinucleate

syncytium which rapidly increases in size and complexity until it gives rise to sporozoites in a terminal episode of cytoplasmic fission. They further suggested that the traditional concept of secondary and tertiary sporoblasts as separate units arising by cytokinesis was based upon Giemsa-stained squashes of salivary gland acini and possibly represented artefactitious fragments of a coherent mass of parasite protoplasm, during specimen preparation. The electron microscopic description of *in situ* sporogonic development of *T. annulata* provided in this thesis, while it is only preliminary, basically agrees with the proposal of Fawcett *et al.* (1982a) that sporogony involves continuous growth and differentiation of a single sporont syncytium. It, however, appears to suggest that the syncytium gives rise to smaller units from which the sporozoites eventually bud off by schizogony.

The fine structural characteristics of *T. annulata* sporozoites described in this study are generally similar to those reported for other members of the phylum Apicomplexa in the class Sporozoea (Scholtyseck and Mehlhorn, 1970; Porchet-Hennere and Vivier, 1971; Scholtyseck, 1979). Within the class, *T. annulata* sporozoites bear a striking similarity to the dissemination forms of *Babesia* parasites, the only major differences being the presence in the latter genus of a subpellicular layer of double membranes (Rudzinska, 1981) and the rarely reported conoid and subpellicular microtubules (Buttner, 1968). The nucleus of *T. annulata* sporozoites, like that of *Babesia* parasites, is non-chromocentric and anucleolate, and differs markedly from that of *Plasmodium* species with distinct heterochromatic clumps and nucleoli (Aikawa, Sterling and Rabbege, 1972).

T. annulata sporozoites have been shown in this study to invade bovine lymphoid cells base first in contradistinction to other sporozoeans such as Babesia (Rudzinska et al., 1976), Eimeria (Jensen and Edgar, 1976), Plasmodium (Bannister, Butcher, Dennis and Mitchell, 1975), Lankesteria (Sheffield et al., 1971), Selenidium (Metais and Schrevel, 1974) and Toxoplasma (Nichols and O'Connor, 1981). In T. parva, Fawcett et al. (1982b) reported that sporozoites entered target lymphoid cells in any orientation. A study of their article, however, shows that in every case where sporozoites were interiorised, the orientation was base first. These authors used a mononuclear-cell enriched fraction from bovine peripheral blood in their study. It is, therefore, possible that some T. parva sporozoites were being phagocytosed by macrophages in a haphazard orientation, hence the misinterpretation. That this is true is supported by the observation by Fawcett et al. (1982b) that even sporozoites in an advanced stage of degeneration could make contact and interiorise, a clear indication of phagocytosis. In a preliminary study in this laboratory by the author using PBL separated from defibrinated blood, T. parva sporozoites made contact with, and entered, target cells base first.

Host lymphoid cell recognition by T. annulata sporozoites is dependent on the presence of receptors on the target cell plasmalemma. The receptors have been shown to be susceptible to lysis by trypsin, and are most likely glycoproteins. Complementary receptors on the basal aspect of T. annulata sporozoites are believed to determine the base-first orientation of the sporozoite consistently

observed in this study. The involvement of receptors in parasite-host recognition and interaction is not unique to T. annulata-bovine lymphocyte system since it is a well established phenomenon in Plasmodium (Miller, 1977; Trigg, 1979) and Babesia (Chapman and Ward, 1977; Jack and Ward, 1981) infections.

The invasion of lymphoid cells and the interiorisation of T. annulata sporozoites is actively achieved by the parasite and is an energy-dependent process which is markedly influenced by temperature. The sporozoites are endowed with intact functional metabolic energy pathways, viz. glycolysis, citric acid cycle and electron transport chain, and thus independently generate the ATP required for their invasive activities culminating in intracellular localisation.

T. annulata sporozoites interiorise by a deepening invagination of the host lymphocyte plasmalemma which remains intact throughout the entry process and only fragments after the parasite is intracellular. The mechanisms underlying the invagination of host lymphocyte plasma membrane are not known. Secretory products of rhoptries have been incriminated in other sporozoeans which invade apex first as reviewed by Aikawa and Kilejian (1979). While Kilejian (1974) and Bannister, Butcher and Mitchell (1977) reported that the products are largely proteinaceous, Garnham, Bird and Baker (1960), Lycke, Norrby and Remington (1968), Rudzinska (1969) and Norrby (1971) believed that rhoptries release enzymatic substances. The role the rhoptries play in the invasion of bovine lymphocytes by T. annulata sporozoites is not clear since the parasite makes contact and interiorises base first, with the rhoptries

well away from the area of contact. Two alternative hypotheses have been proposed in connection with mechanisms of host plasmalemmal invagination: one suggests that shape changes are caused by energy-dependent alterations in the protein, such as spectrin, to which the erythrocyte membrane is attached (Hayashi, Plishker, Vaughan and Penniston, 1975); the other proposes that unequal expansion of the two lipid leaflets of the membrane bilayer could cause inward or outward bending (Sheetz and Singer, 1974; Feo and Mohandas, 1977). According to the latter proposal, amphiphilic, positively charged molecules inserted into the inner membrane leaflet of the erythrocyte lead to its inward bending and thus form endocytic vacuoles. In this thesis, it has been shown that T. annulata sporozoites form pellicular projections. It is possible that such localised pellicular distortions, following a firm parasite-lymphocyte membrane attachment, during active entry by a sporozoite could result in the invagination of host lymphocyte plasma membrane.

The interiorised T. annulata sporozoites, while they dedifferentiate into trophozoites, feed and transform into schizonts, are concomitantly subjected to host lysosomal activity. Viable developing trophozoites do not fuse with lysosomal vesicles, thereby circumventing enzymatic digestion. Failure of lysosomal fusion is attributed to structural alterations accompanying the transformation of interiorised T. annulata sporozoites into trophozoites. In malaria parasites, the investing host erythrocyte membrane acquired by merozoites during invasion is not shed throughout the intra-erythrocytic development of the parasite. Comparative morphological

and cytochemical studies (Seed, Aikawa, Sterling and Rabbege, 1974; Langreth, 1977) of the vacuole membrane and normal erythrocyte membrane indicated differences in their surface charge, glycoprotein and enzyme distribution. Langreth (1977) concluded that the vacuole membrane, although derived from erythrocyte membrane, had been altered by its association with the developing malaria parasite. The alteration of host membrane precludes its fusion with lysosomes (Aikawa and Kilejian, 1979).

Developing intracellular Theileria parasites provoke blastoid transformation of host lymphoid cells by insinuating gene fractions of their DNA. A symbiotic relationship is consequently established between the parasite and the host lymphocyte, the latter replicating at a rate conducible to parasite multiplication. Such transformed cells, although susceptible to superinfection by a homologous Theileria species, cannot support the establishment of such interiorised sporozoites as schizonts. The interference is believed to be achieved by the incumbent schizont by blocking host cell gene sites. The influence of Theileria on host replicative machinery is illustrated in a lymphosarcomatous cell line (BL-20) superinfected by T. annulata where the parasite appears to take over the control mechanisms.

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Appendix Table 1 The distribution of T. annulata sporozoites in 40 target lymphocytes with increasing time of incubation

Number of sporozoites per lymphocytes	Number of lymphocytes with x number of T. annulata sporozoites																													
	15 minutes								3 hours								18 hours													
1	11	8	20	15	14	16	19	23	3		2	4	1	2	1	1	1	1		1										
2	12	14	10	18	11	11	13	8	7	6	5	5	2	5	4	2	3		2	2	2		2	2						
3	15	10	8	4	6	9	4	3	7	6	6	10	7	1	4	5	2	3	3	1	1	1	1	2						
4	1	6	1	3	7	2	3	2	7	10	4	6	8	5	2	5	3	6		1	4	2	2	3						
5	1	1	1				1	2	7	3	5	6	7	7	14	5	2	3	3	2	2	3	5	7						
6		1			2	1		2	1	6	3	5	4	7	3	7	4	1	5	3	5	4	7	4						
7										1	5		2	2	3	7	5	4	3	4	4	2	2	1						
8						1			3	3	5	3	3	1	3	2	6	4	6	3	2	6	6	4						
9									2		1		1	2	2	2	4	3	4	6	4	3	2	3						
10										1	2		1	3	3	2	6	3	3	4	3	3	3	1						
11									2		2	1		5	1	1	1	1	4	4	2	6	4	4						
12										1			2				1	4		2	2	3	3	1						
13									1	1			2				1	1	1	1	3	4	1	2						
14																	1	3	1	2	2		1	2						
15										2						1		1	4	1	2									
16																					1			2						
17																		1						2						
18																		1	1		1	1	1							
19																														
20																				2		2								
21																				1										

Appendix Table 2 Total cell number per culture well

Treatments	Day 0	Day 3	Day 6	Day 9	Day 12
A1	4×10^6	2.9×10^5	1.2×10^6	5.0×10^5	6.5×10^5
A1	4×10^6	3.4×10^5	1.2×10^6	8.0×10^5	7.0×10^5
A1	4×10^6	3.4×10^5	1.4×10^6	7.3×10^5	6.8×10^5
A1	4×10^6	2.3×10^5	9.5×10^5	6.8×10^5	9.0×10^5
A3	4×10^6	2.2×10^5	1.2×10^6	2.5×10^5	1.1×10^5
A3	4×10^6	2.9×10^5	1.1×10^6	3.0×10^5	1.0×10^5
A3	4×10^6	3.2×10^5	1.1×10^6	2.5×10^5	1.2×10^5
A3	4×10^6	3.2×10^5	1.1×10^6	2.9×10^5	1.8×10^5
B4	1×10^5	2.7×10^5	2.0×10^6	2.7×10^6	4.5×10^6
B4	1×10^5	2.7×10^5	2.1×10^6	2.3×10^6	3.5×10^6
B4	1×10^5	2.3×10^5	1.5×10^6	2.5×10^6	4.3×10^6
B4	1×10^5	2.7×10^5	1.9×10^6	2.2×10^6	3.5×10^6
B6	1×10^5	1.9×10^5	1.8×10^6	3.0×10^6	4.0×10^6
B6	1×10^5	2.4×10^5	1.8×10^6	1.7×10^6	3.5×10^6
B6	1×10^5	2.2×10^5	2.4×10^6	2.5×10^6	4.5×10^6
B6	1×10^5	2.0×10^5	1.7×10^6	2.2×10^6	1.9×10^6
C7	1×10^5	1.6×10^5	1.5×10^6	3.3×10^6	2.4×10^6
C7	1×10^5	1.9×10^5	1.3×10^6	1.9×10^6	2.5×10^6
C7	1×10^5	1.7×10^5	2.2×10^6	2.5×10^6	3.3×10^6
C7	1×10^5	1.4×10^5	1.5×10^6	1.7×10^6	3.8×10^6
C9	1×10^5	2.0×10^5	1.8×10^6	2.4×10^6	2.6×10^6
C9	1×10^5	1.8×10^5	1.3×10^6	2.3×10^6	2.8×10^6
C9	1×10^5	1.3×10^5	1.6×10^6	2.3×10^6	2.4×10^6
C9	1×10^5	1.4×10^5	1.8×10^6	2.6×10^6	2.6×10^6
D10	2×10^5	2.8×10^5	4.3×10^6	5.8×10^6	1.1×10^7
D10	2×10^5	5.0×10^5	4.3×10^6	6.0×10^6	9.8×10^6
D10	2×10^5	2.7×10^5	4.0×10^6	5.0×10^6	9.0×10^6
D10	2×10^5	3.0×10^5	4.3×10^6	5.0×10^6	6.3×10^6
D12	2×10^5	2.6×10^5	2.8×10^6	3.5×10^6	1.1×10^7
D12	2×10^5	2.7×10^5	1.8×10^6	3.8×10^6	1.2×10^7
D12	2×10^5	2.7×10^5	3.0×10^6	6.3×10^6	1.1×10^7
D12	2×10^5	3.0×10^5	3.8×10^6	7.1×10^6	1.1×10^7

Appendix Table 3 Mean schizont nuclear number per 50 infected cells/replicate culture

Treatments	Day 3	Day 6	Day 9
A1	5.8	10.5	15.6
A1	6.3	8.8	14.2
A1	7.7	9.1	16.0
A1	6.6	10.3	12.5
B4	16.4	12.4	15.4
B4	16.9	12.0	13.4
B4	12.2	13.4	13.2
B4	13.1	12.3	13.7
B6	14.1	13.2	14.4
B6	14.9	10.8	12.7
B6	13.9	11.7	12.4
B6	13.7	12.5	13.8
C7	13.8	11.4	13.6
C7	14.2	12.7	14.6
C7	13.9	13.2	13.7
C7	13.3	11.3	15.1
C9	13.8	12.3	14.7
C9	14.1	11.9	13.0
C9	13.8	10.8	12.6
C9	13.5	14.0	15.8
D10	10.1	8.2	9.7
D10	11.7	8.7	9.0
D10	10.8	9.3	10.2
D10	10.8	10.0	12.7
D12	12.1	9.8	10.0
D12	10.1	8.0	10.2
D12	9.0	11.3	9.1
D12	11.6	8.7	9.5

Appendix Table 4 The effect of metabolic energy inhibitor drugs on the number of T. annulata sporozoites invading bovine lymphocytes/1,000 cells

Reagent	Replicates	C^1	C^2	Molar concentrations of inhibitor drugs				
				10^{-2}M	10^{-3}M	10^{-4}M	10^{-5}M	10^{-6}M
Lithium iodoacetate	1)	776	594	30	72	149	457	504
	2)	825	808	27	42	89	294	549
	3)	643	787	49	66	103	335	540
	4)	565	521	54	53	100	238	337
Sodium fluoride	1)		560	37	75	267		
	2)		738	44	77	344		
	3)		743	46	100	315		
	4)		354	40	121	262		
Potassium cyanide	1)		734	95	349	639	916	789
	2)		696	197	443	401	537	625
	3)		844	212	407	649	633	802
	4)		942	147	476	430	816	655
Antimycin A	1)		497		104	143	325	391
	2)		510		128	249	150	244
	3)		385		105	292	350	328
	4)		465		130	129	257	302
2,4-Dinitro-phenol	1)		719	27	81	216	554	646
	2)		680	31	74	260	375	552
	3)		660	18	111	279	520	560
	4)		840	9	91	256	381	610

[1]Control cultures set up by mixing T. annulata, kept at 4°C and warmed to 20°C, with PBL

[2]Control cultures set up by mixing T. annulata, pre-warmed to 37°C for 3 hours, with PBL

Appendix Table 5 Cell concentration/ml

Cell line	Day 0	Day 1	Day 2	Day 3
BL-20	2×10^5	2.88×10^5	4.63×10^5	9.75×10^5
	2×10^5	2.60×10^5	4.75×10^5	8.50×10^5
	2×10^5	3.33×10^3	4.45×10^5	9.0×10^5
	2×10^5	3.00×10^5	5.25×10^5	8.68×10^5
BL-20-T.a.(H)	2×10^5	3.68×10^5	1.23×10^6	2.26×10^6
	2×10^5	4.53×10^5	1.17×10^6	2.47×10^6
	2×10^5	3.20×10^5	1.07×10^6	2.30×10^6
	2×10^5	3.65×10^5	1.11×10^6	2.10×10^6
BL-20-T.a.(A)	2×10^5	5.43×10^5	1.40×10^6	2.51×10^6
	2×10^5	5.70×10^5	1.39×10^6	2.47×10^6
	2×10^5	4.38×10^5	1.20×10^6	2.62×10^6
	2×10^5	4.40×10^5	1.30×10^6	2.56×10^6
PBL-T.a.(H)	2×10^5	3.98×10^5	1.21×10^6	1.88×10^6
	2×10^5	6.78×10^5	1.24×10^6	1.53×10^6
	2×10^5	6.23×10^5	1.52×10^6	1.52×10^6
	2×10^5	6.18×10^5	1.03×10^6	1.48×10^6
PBL-T.a.(A)	2×10^5	4.15×10^5	7.68×10^5	1.60×10^6
	2×10^5	4.03×10^5	7.88×10^5	1.58×10^6
	2×10^5	4.35×10^5	6.70×10^5	1.56×10^6
	2×10^5	4.20×10^5	7.78×10^5	1.39×10^6

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FINE STRUCTURE AND INVASIVE BEHAVIOUR OF THE EARLY DEVELOPMENTAL STAGES OF *THEILERIA ANNULATA* IN VITRO

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ABSTRACT

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The interaction, in vitro, between bovine peripheral blood lymphocytes and sporozoites of *Theileria annulata* (Ankara) was studied by light and electron microscopy. Beginning five minutes following incubation, samples were taken for Giesma-stained smears and glutaraldehyde-fixed pellets, for light and electron microscopy, respectively.

Sporozoites of *T. annulata* measure an average of 0.9 μm long, 0.8 μm broad and possess a limiting unit membrane, the pellicle; a round-to-ovoid, eccentrically situated, non-chromocentric nucleus; double-membraned, tubular, acristate mitochondria; varying numbers of anisocytic, densely osmiophilic and pleomorphic organelles, the rhoptries which together with the polar ring form the apical complex; and numerous, loosely scattered, electron-dense ribosomal particles. As early as 5 min of incubation, sporozoites had made contact with, and penetrated, lymphocytes. Sporozoites consistently attached to the lymphocyte plasmalemma by their basal end, possibly at specific receptor sites. Apparently only a proportion of lymphocytes (up to 40% and more commonly 10—20%) were susceptible. Two subpopulations of the susceptible lymphocytes were observed; one which appeared to have receptor sites localized on one pole of the plasmalemma and the other subpopulation in which the receptor sites were distributed evenly around the plasmalemmal surface. Within individual susceptible lymphocytes, the number of interiorized sporozoites increased from 1 to 3 at 5—10 min to as many as 15 or more parasites at around 60 min of incubation. *Theileria annulata* sporozoites were interiorized by the invagination of the host cell plasmalemma which remained intact throughout the process but later fragmented. Within 30 min of interiorization, each sporozoite underwent dedifferentiation by the loss of its rhoptries and transformed into a trophozoite. Around 24 h, the trophozoite, a uninucleate, motile and feeding stage of the parasite, developed into a schizont by an acentric, closed mitosis.

INTRODUCTION

The entry of parasites into cells is a crucial step in the pathogenesis of an infection. The precise knowledge of the initial events that characterize host

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target cell—parasite interaction is, consequently paramount in the development of protective measures against disease entities. In animal diseases caused by protozoan parasites of the genus *Theileria*, little is known about the mechanism by which sporozoites, the stage in the salivary glands of the arthropod vector ticks, infective for their various bovine, ovine and caprine vertebrate hosts, enter the host target cells and the morphological changes they undergo before developing into the multinucleate, intralymphocytic stage, the schizont. As a prerequisite to the interiorization of intracellular parasites, such as *Theileria* species, contact must first be made with the host cell of predilection. The contact may either be made haphazardly as in the case of flagellates (Miller and Twohy, 1967; Akiyama and Haight, 1971; Lewis, 1974; Alexander, 1975) or in a particular orientation as in the case of merozoites of *Babesia* (Rudzinska et al., 1976) and *Plasmodium* (Miller et al., 1973; Dvorak et al., 1975), and sporozoites of *Lankesteria culicis* (Sheffield et al., 1971) in which the anterior end of the parasites usually attach to the host cell membrane. The latter case would incriminate specific reciprocal recognition sites on the parasite limiting membrane and host cell plasmalemmal surfaces. Interiorization may be achieved by disrupting the host cell plasma membrane as do the members of the microsporidian genus *Nosema* (Weidner, 1972, 1976), by active invasion and invagination of the host cell plasmalemma without disrupting its continuity e.g., *Selenidium hollandei* (Metais and Schrevel, 1974), *Eimeria magna* (Jensen and Hammond, 1975; Jensen and Edgar, 1976) and mammalian malaria parasites, *Plasmodium berghei* and *P. knowlesi* (Danforth et al., 1980) or mainly by the phagocytic activity of the host cell as for amastigotes of *Leishmania donovani* (Chang and Dwyer, 1978; Pearson et al., 1981) and *L. mexicana* promastigotes and *Trypanosoma cruzi* trypomastigotes and promastigotes (Alexander, 1975; Nogueira and Cohn, 1976). Observations on the interaction between *Theileria parva* and bovine peripheral blood lymphocytes in vitro have been based on studies by light and interference contrast microscopy (Brown et al., 1973; Stagg et al., 1981). Although the latter authors alluded to the presence of a pre-schizont stage, no mention was made about the orientation of the sporozoite during its contact with the host cell, whether the host cell plasmalemma was disrupted or not, how and when the pre-schizont developed from the sporozoite and whether they are structurally different, or the characteristics of the pre-schizont and how it eventually developed into a schizont. In the first preliminary ultrastructural study of the early stages of *Theileria annulata* in vitro (Jura, 1981), a brief description of the salient features of the penetration process was given and the term trophozoite was introduced for the first time to describe the uninucleate, intralymphocytic stage in the life cycle of *Theileria* which develops from the sporozoite by a dedifferentiation process and which, through growth and nuclear division, gives rise to the schizont stage.

The present study pays special attention to the fine structure of *T. annulata* sporozoites with particular reference to the distribution of the rhoptries,

and also describes the sequential changes of the sporozoite in the target host cell.

MATERIALS AND METHODS

Isolation of Theileria annulata *sporozoites*

Methods for the isolation of sporozoites and lymphocytes were modified after Brown (1979). Forty adult *Hyalomma anatolicum anatolicum* ticks infected with the Ankara strain of *Theileria annulata* and fed on rabbit ears for 3 days to allow maturation of sporozoites were surface sterilized by three different treatments: (a) three times in 1% Benzalkonium chloride (Roccal antiseptic, Winthrop Laboratories, Surrey, England), (b) three times in 70% (v/v) ethanol, (c) four times in Eagle's minimum essential medium (MEM) with Hanks' salts (Gibco-Biocult, 3 Washington Road, Paisley, PA3 4EP, Scotland), containing 200 iu/ml Benzyl Penicillin (sodium) B.P. 1 000 000 iu (Glaxo Laboratories Ltd., Greenford, England), 200 μg/ml streptomycin sulphate B.P. 1g (Glaxo Laboratories Ltd., Greenford, England) and 100 u/ml Nystatin B.P. 500 000 units (Mycostatin, E.R. Squibb & Sons Ltd., Twickenham, Middlesex, England). The medium in the final wash was discarded and 2.5 ml of 3.5% bovine plasma albumin in MEM (MEM/BPA — Bovine Plasma Albumin — Armour Fraction V, Sigma Chemicals) containing 200 iu/ml Benzyl Penicillin, 200 μg/ml. Streptomycin sulphate and 100 u/ml Nystatin were added and the ticks transferred to a sterile mortar. They were ground thoroughly with a sterile pestle (4 rounds), each time adding and recovering 2.5 ml of MEM/BPA 3.5% as above so that, finally, 10 ml of sterile ground up tick supernate (GUTS) were obtained (i.e., 4 tick equivalents/ml). The GUTS was centrifuged at 100 *g* for 5 min (1000 rpm MSE Minor) and the supernate containing sporozoites aseptically recovered into a 20-ml sterile universal bottle. Further removal of the debris was done by passing the GUTS through a sterile 47 mm Millipore Swinnex filter, with AP25 prefilter and 8 μm MF filter (Millipore Corporation, Bedford, MA, U.S.A.). Fifty μl filtered GUTS were centrifuged at 700 rpm for 10 min (Cytospin Centrifuge; Shandon Southern Instrument Ltd., Frimley Road, Camberley, Surrey, England) onto microscope slides and smears stained with Giemsa stain to check for the presence, the morphology and the concentration of sporozoites in the suspension. Sporozoite pellets for electron microscopy were obtained by centrifuging GUTS at 1500 *g* for 15 min.

Separation of bovine peripheral blood lymphocytes

Fifty ml jugular blood, anticoagulated in 10 ml acid citrate dextrose (Fenwall Division, Travenol Laboratories Ltd., Thetford, Norfolk, England), were centrifuged at 2250 *g* for 30 min (3000 rpm MSE Mistral 2L at 5°C) to obtain buffy coat. The buffy coat cells were recovered in about 3 ml and dilu-

ted in 9 ml phosphate buffered saline, PBS, pH 7.3 (Dulbecco A). The diluted buffy coat cell suspension was carefully layered onto 8 ml Ficoll/sodium diatrizoate gradient, Sp. gr. 1.07 (Ficoll-paque, Pharmacia-Fine Chemicals AB, Uppsala, Sweden) and the interface subjected to 670 *g* for 40 min (2000 rpm MSE Mistral 2L at 20° C). The interface cells were recovered in about 5 ml and washed twice in PBS, pH 7.3 (20 ml) at 275 *g* (1450 rpm MSE Mistral Minor for 10 min, then 5 min). The cell pellet was resuspended in 10 ml growth medium — RPMI 1640/20% fetal calf serum (FCS) with 100 iu/ml Benzyl Penicillin (sodium) B.P., 100 μg/ml streptomycin sulphate B.P. and 2 mM/ml L-glutamine, 200 mM (100 ×) (Gibco-Biocult). The cell concentration was adjusted to 8×10^6 cells/ml of growth medium.

Establishment of cultures

Cultures were established in tissue culture Cluster plates (Costar, 205 Broadway, Cambridge, MA). 0.25 ml cell suspension (2×10^6 cells) in growth medium, and 0.25 ml sporozoite suspension were seeded into each of the 12 wells used. The cultures were put immediately in a humidified plastic box, gassed with 5% CO_2 /95% air and incubated at 37° C.

Sampling

Samples were obtained at 5, 10, 20, 30, 40 and 60 min, and 2, 3, 6, 18, 24 and 48 h of incubation. For Giemsa-stained smears a 50 μl aliquot of thoroughly mixed culture suspension was centrifuged at 700 rpm for 10 min (Cytospin; Shandon Southern Instruments). After obtaining samples for light microscopy at each stage, the remainder of the culture was aspirated, made up to 5 ml with warm growth medium (37° C) and centrifuged at 275 *g* for 10 min (1450 rpm MSE Minor at 20° C). The pellet was resuspended in 5 ml serum-free RPMI 1640 (37° C) and spun at 275 *g* for another 10 min. The resultant pellet was processed for transmission electron microscopy.

Transmission electron microscopy

Pellets were fixed overnight at 4° C in 2.5% cacodylate-buffered glutaraldehyde (TAAB Laboratories, Reading, England), washed several times in cacodylate buffer, pH 7.4 and post fixed for 2 h in 1% osmium tetroxide (Johnson-Mathey, London, England). After 3 changes in cacodylate buffer, samples were dehydrated in 10, 50, 90% and absolute ethanol (2 treatments, 15 min each time in each concentration of ethanol). They were then cleared twice, 15 min each time in propylene oxide (Fisons, Loughborough, England), embedded in araldite (Agar Aids, Stanstead, Essex, England) and polymerized at 60° C for 2 days. Sections were cut with LKB glass knives (LKB, Stockholm, Sweden) on a Cambridge—Huxley ultramicrotome, mounted on copper grids and stained with uranyl acetate (Watson, 1958) and lead citrate

(Reynolds, 1963). Sporozoite pellets and whole tick salivary glands, obtained by dissecting out cuticular covering in growth medium, for ultrastructural studies were fixed in 2.5% glutaraldehyde above overnight and had the same postfixation treatment as the pellets. Sections were examined on a Philips 400 electron microscope.

RESULTS

Infective Theileria annulata *sporozoites*

Infective sporozoites, the form of the parasite which invades vertebrate host lymphoid cells and initiates the developmental cycle, were usually ovoid, measured on average about 0.9 μm long, 0.8 μm broad and were surrounded by a thin, limiting unit membrane, the pellicle, 80—100 Å thick (Fig. 1). The sporozoites of *T. annulata* do not possess a surface coat and have no additional supporting membranes on the inner aspect of the pellicle apart from the polar ring. Situated on the basal aspect of the sporozoite is a round to

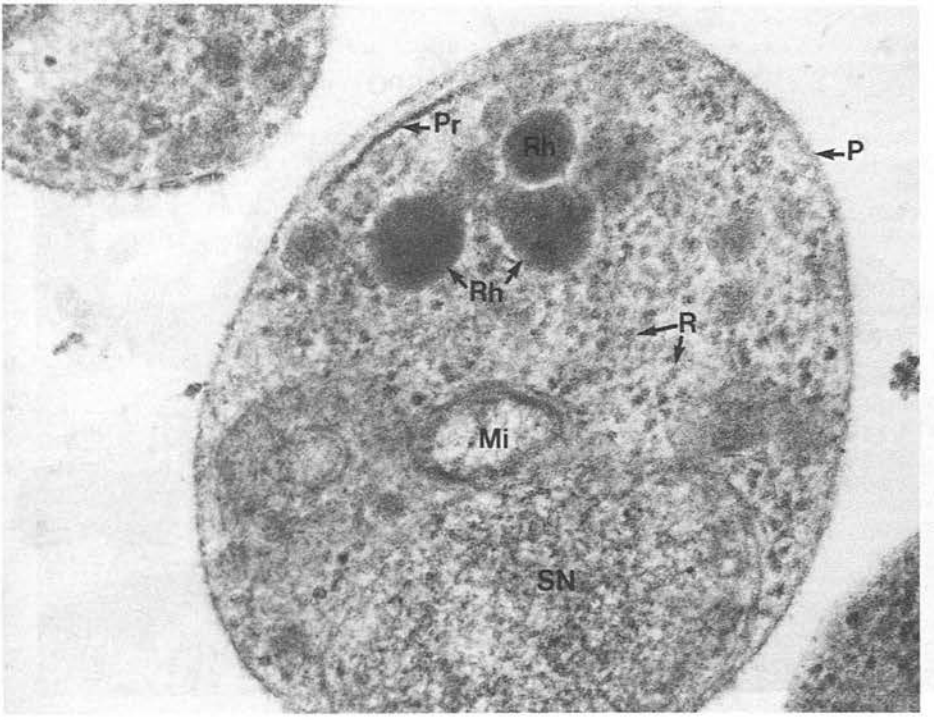

Fig. 1. *Theileria annulata* sporozoite showing fine structural characteristics. The apical complex consists of several rhoptries (Rh) and a polar ring (Pr). The sporozoite is surrounded by a unit membrane, the pellicle (P), and contains in the ground substance of its cytoplasm, numerous ribosomes (R), and acristate mitochondria (Mi). The nucleus (SN) is eccentric and non-chromocentric. Magnification × 115 470.

ovoid nucleus encased in a nuclear envelope composed of two membranes, each 75 Å thick. The outer nuclear membrane is patchily covered by ribonucleoprotein particles, 150—250 Å diameter. The nucleoplasm contains no nucleoli and shows no distinct organisations of chromatin fibrils. It consists of uniformly distributed, closely packed ribonucleoprotein-like particles, 75—200 Å diameter. The ground substance of the cytoplasm contains numerous, loosely scattered, electron-dense ribosomal particles, 150—250 Å diameter. Mitochondria are acristate, tubular and are enclosed by two membranes. The inner membrane is thicker and measures 100 Å while the relatively thinner outer membrane is 60 Å thick. The intra-membranous space is lucent and measures about 40 Å in thickness. The shape of the mitochondria may be round, curved, dumb-bell, elongate or may resemble a pear while the diameter of luminal space may range from 0.02—0.33 μm. The rhoptries, densely osmiophilic organelles, are anterior to the nucleus and permeate the entire cytoplasm of the sporozoites. They assume different shapes and sizes depending on the plane of section. A radial arrangement around what appears to

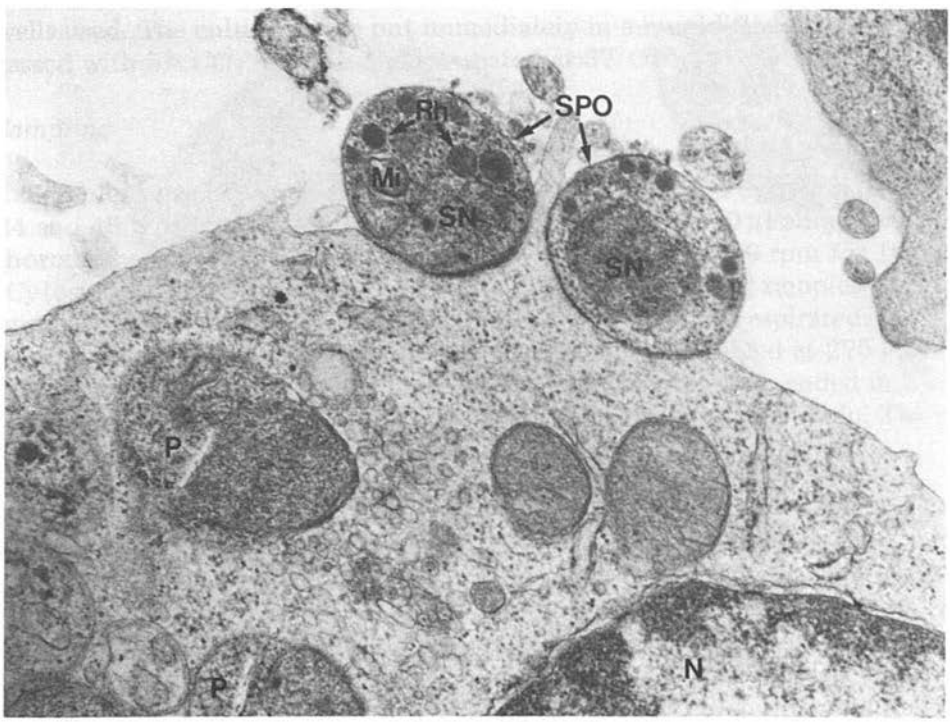

Fig. 2. An early stage of invasion illustrating multiple infection and attachment. Each of the sporozoites (SPO) has made contact with the host plasma membrane by the aspect of its pellicle adjacent to its nucleus (SN). The anterior end of the sporozoite comprising the rhoptries (Rh) and mitochondria (Mi) is away from, and does not come in contact with, lymphocyte plasmalemma. Two parasites (P) are already within the cytoplasm of the target lymphocyte. Magnification × 30 350.

be a central canal was manifest in many sections. Several rhoptries together with the polar ring represent the apical complex in *T. annulata* sporozoites (Fig. 1).

Attachment to, and invasion of, bovine lymphocytes

Sample fixed at 5 min of incubation (Fig. 2) shows *T. annulata* sporozoites at various stages of invasion. At this early stage the sporozoites still possess their characteristic ovoid shape and the specialized, densely osmiophilic organelles, the rhoptries. The parasites consistently made contact with the plasmalemma of the host lymphocyte by their basal end adjacent to the nucleus. Only a proportion of the lymphocytes (up to 40% and more commonly 10–20%) were susceptible. Two groups of susceptible lymphocytes were observed: one group in which sporozoite entry localized on one pole of the plasma membrane while the other subpopulation of cells had sporozoites distributed uniformly around the plasmalemmal surface (Fig. 3). Within in-

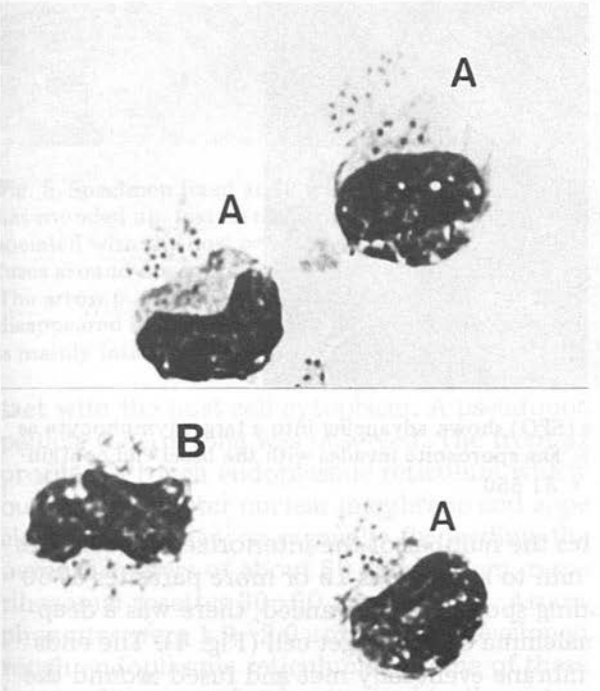

Fig. 3. A smear prepared at 1 h of incubation showing two different groups of susceptible bovine lymphocytes. A, which are invaded by sporozoites from one pole of the plasma membrane and B, in which the penetration is uniform around the lymphocyte plasmalemmal surface. Magnification × 1 280.

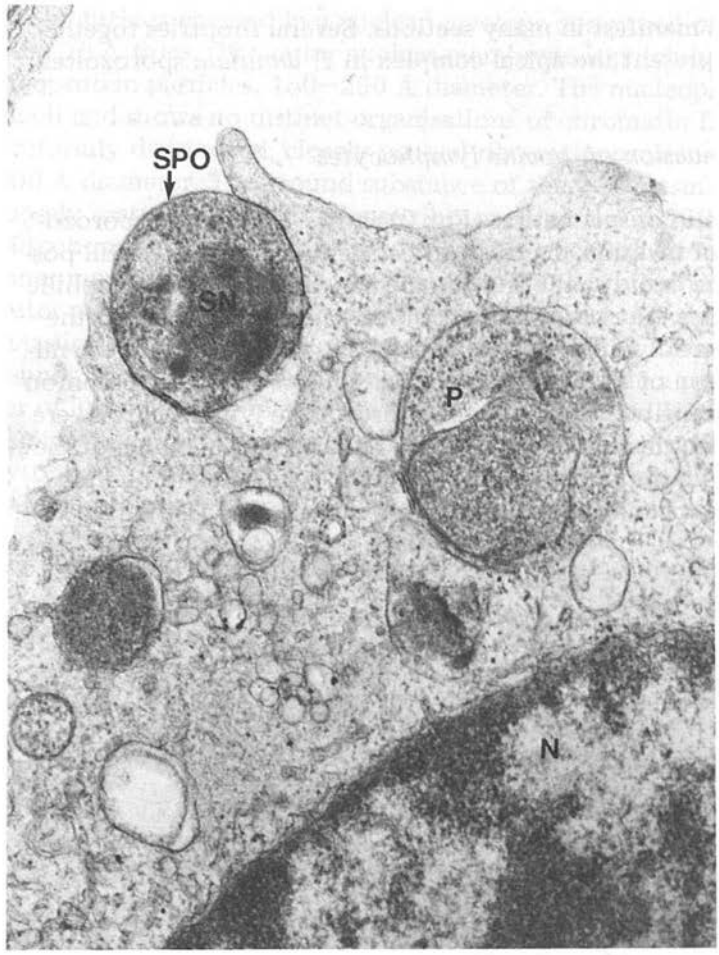

Fig. 4. *Theileria annulata* sporozoite (SPO) shown advancing into a target lymphocyte as the latter's plasmalemma invaginates. The sporozoite invades with the basal end containing the nucleus (SN). Magnification × 31 650.

dividual susceptible lymphocytes the number of the interiorized sporozoites increased from 1 to 3 at 5—10 min to as many as 15 or more parasites at 60 min of incubation. As the invading sporozoites advanced, there was a deepening invagination of the plasmalemma of the target cell (Fig. 4). The ends of the invaginated host cell membrane eventually met and fused around the sporozoite. The invading parasites occupied the juxtanuclear position of the lymphocytes within 30 min of incubation, became roundish, completely lost their rhoptries and transformed into a new stage in the life cycle of the parasite, the trophozoite (Fig. 5). The surrounding host cell membrane which remained intact throughout the interiorization process was seen to fragment and disappear progressively so that the trophozoite remained in direct con-

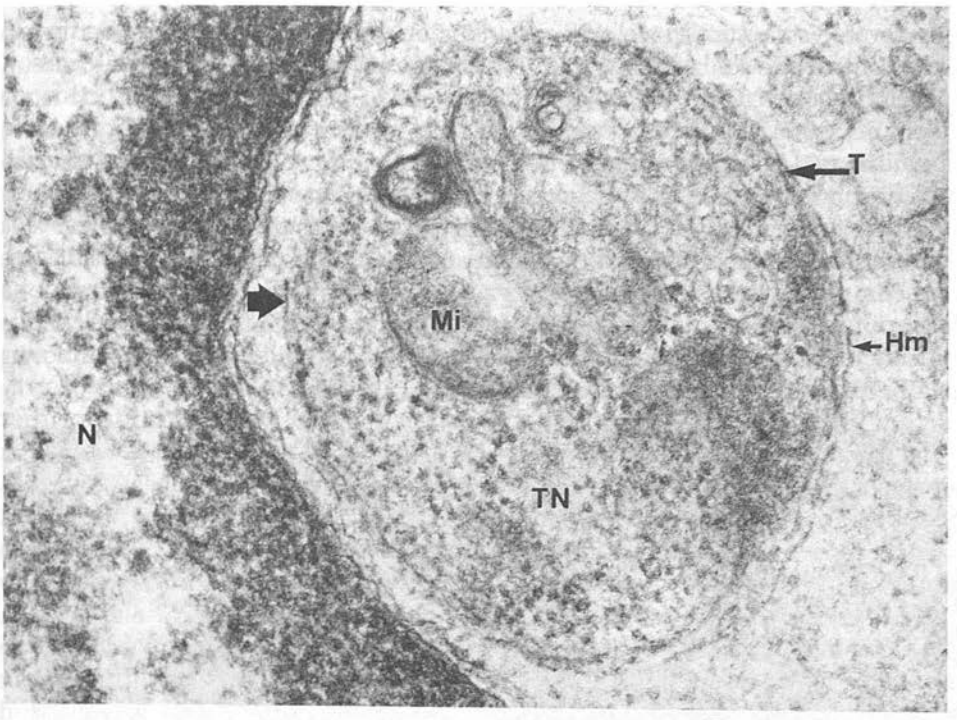

Fig. 5. Specimen fixed at 30 min of incubation. The interiorized *T. annulata* sporozoite has rounded up, lost its rhoptries and transformed into a trophozoite (T) seen closely associated with the host cell nucleus (N). At this stage the host cell membrane (Hm) which fuses around the sporozoite during interiorization is seen fragmenting and disappearing. The arrow (➡) depicts a fragment of the Hm. The membrane has completely disappeared in the anterior aspect of the trophozoite close to the mitochondria (Mi), but is mainly intact towards the trophozoite nucleus (TN). Magnification × 93 170.

tact with the host cell cytoplasm. A pseudopod extension of the trophozoite pellicle 0.5 μm long was observed. The trophozoites developed increasing profiles of rough endoplasmic reticulum which were in some cases continuous with the outer nuclear membrane and appeared as vesicles, coils or flat, elongate cisternae; an organelle for feeding, the cytostome, with inner and outer diameters of about 60 and 107 nm, respectively; and large numbers of ribosomal rosettes 30—60 nm diameter. Around 24 h the majority of trophozoites were 1.9—2.0 μm and had developed prominent polysomes and rough endoplasmic reticulum. In some of these parasites an organelle of unknown function, closely attached to the outer nuclear membrane which we have called the perinuclear organelle was seen. Even at this stage of development variations in the size of individual trophozoites was marked.

Trophozoite to schizont

After its transformation from the sporozoite, the trophozoite underwent

considerable structural development and some increase in size. Imminent development of the schizont stage of *Theileria* from the trophozoite was hallmarked, between 18—24 h, by the appearance of a bundle of spindle microtubules 300—500 Å diameter (Fig. 6). The microtubules were wholly intranuclear and have been seen to have their axes, not in the centrioles but, in the amorphous, osmiophilic plaque-like structures associated with both inner and outer nuclear membranes, the spindle pole bodies (SPB). The nuclear membranes remained intact and chromosome uncoiling and condensation were completely in abeyance. By this acentric, closed mitosis with intranuclear spindle microtubule formation, a binucleate schizont results. Each schizont nucleus underwent similar division to produce a multinucleate schizont, usually referred to as the macroschizont.

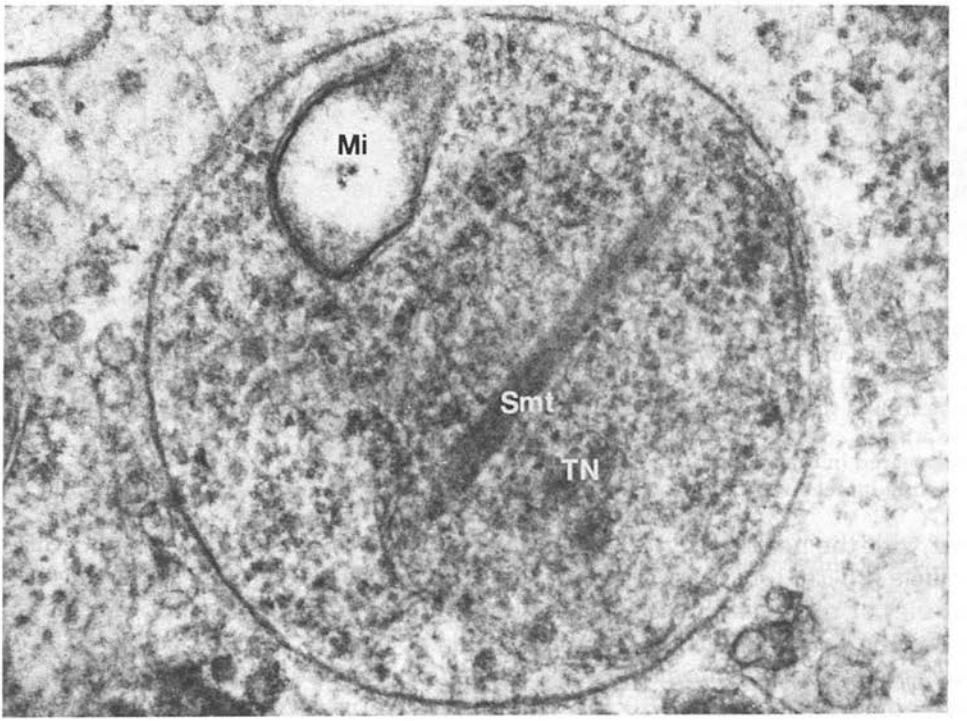

Fig. 6. At 24 h of incubation, a trophozoite manifests well developed anisotropic, intranuclear spindle microtubules (Smt) depicting imminent division of the trophozoite nucelu to produce a schizont. Magnification × 91 460.

DISCUSSION

In this investigation, we have demonstrated that prior to interiorization, *Theileria annulata* sporozoites must come into contact with the plasmalemma of the target bovine lymphocytes in a particular orientation. They consistently attached to the host cell plasmalemma by their basal end. This ob-

servation implicates specific receptor sites on the plasmalemma of the target host cell and reciprocal recognition sites on the basal aspect of *T. annulata* sporozoites. The availability of such surface determinants in the lymphocyte plasma membrane would influence its susceptibility to infection while their distribution would determine the pattern of attachment of the sporozoites to the cell surface. In our study, only a proportion of the lymphocytes were susceptible to infection and two subpopulations of susceptible cells were observed: one in which sporozoites penetrated at one pole of the lymphocyte and the second group in which sporozoites attached and penetrated more evenly around the plasmalemma. It would, therefore, be reasonable to suggest that the susceptible bovine lymphocytes possessed reciprocal receptor sites to those on the basal aspect of the sporozoites, and that the two patterns of sporozoite attachment observed in this study were related to the distribution of receptor sites on the host lymphocyte plasma membrane. The observations in *Theileria parva* infection that only 25% of the cells (Stagg et al., 1981), and that only a discrete subpopulation of T lymphocytes (Pinder et al., 1981), are susceptible to transformation by the parasite corroborate our observations. The ability of haemosporozoans of the genera *Plasmodium* and *Babesia* to attach to, and subsequently penetrate, mammalian erythrocytes is also directly related to their ability to recognize specific determinants on the host cell membrane. Individual species of *Plasmodium* are highly host-specific (Garnham, 1966). The specificity of plasmodial—erythrocyte interactions has been shown to be related to surface components on the red cell membrane (McGhee, 1953; Butcher et al., 1973; Miller et al., 1973). Whereas *Plasmodium knowlesi* parasites interact with Duffy blood groups (Miller et al., 1975), *Plasmodium falciparum* merozoites appear to interact with a glycoprotein on the erythrocyte surface (Miller et al., 1977). In *Babesia rodhaini* infection, receptors dependent on C3b component of the complement have been shown to be necessary for the penetration process (Chapman and Ward, 1977).

It has also been demonstrated in this study that *T. annulata* sporozoites penetrate bovine lymphocytes by invaginating the plasma membrane of the host cell and that the plasmalemma is not disrupted during the penetration process. The host cell membrane surrounding the parasite fragments and is lost much later when the invading parasite is within the cytoplasm of the host cell. The exact mechanism involved in the interiorization of the sporozoite is not yet fully understood, but has been shown in this laboratory to be an active process which is temperature-dependent. *Theileria annulata* sporozoites differ from any other haemosporozoans so far studied in that they attach and interiorize by their basal ends, i.e., away from the rhoptries. The significance of this observation is considered in greater detail in a subsequent communication (Jura et al., 1983) but is believed to facilitate the early release of the sporozoite genomic factors that confer on the *Theileria*-infected lymphocytes the ability to grow continuously in culture. That theilerial schizont per se may be responsible for the continuous lympho-blastoid transfor-

mation of their host lymphoid cells has been suggested (Malmquist et al., 1970).

The characteristics of the trophozoite (Jura, 1981) are emphasized. It is shown that a *T. annulata* sporozoite rounds up, loses its rhoptries and thus transforms into a trophozoite which feeds through its cytostome by phagotrophy, moves by pseudopod extensions of its pellicle and whose single nucleus divides around 18-24 h and develops into a schizont with more than one nucleus. During the division of the trophozoite nucleus, there is no condensation of chromosomal material. The lack of chromosomal uncoiling and condensation is similarly observed in *Achyla bisexualis* (Griffin et al., 1974) in which this phenomenon, at mitosis, is the result of a short nuclear cycle time, i.e., less than 51 min, suggesting that it may be inefficient, and perhaps impossible, to orchestrate chromatin condensation in such a short time.

From a series of electron micrographs studied in this investigation it became evident that the densely osmiophilic structures that assume tear drop, club, round or ovoid shapes and appear in a variety of sizes within the cytoplasm of *T. annulata* sporozoites represent one organelle, the rhoptries. We believe that the marked pleomorphism and size differences are attributable to the plane of section. In many instances up to six or more rhoptries were observed to converge around a central structure as if they were emptying their contents into a ductule. The entire complex system would seem to terminate just beneath the pellicle in the form of an apical complex. The view that the large (rhoptries) as well as the small (micronemes) structures in the anterior half of the parasite might be functional or evolutionary stages of the same complex of organelles has been expressed for *Toxoplasma gondii* (Sheffield and Melton, 1968; Vivier and Petitprez, 1972) and for *Eimeria callospermophili*, *E. falciformis*, *Babesia bigemina*, *B. ovis* and *Toxoplasma gondii* (Scholtyseck and Mehlhorn, 1970). The large elements might only be ductules of a greater tubular system.

CONCLUSIONS

Theileria annulata sporozoites, like the other members of the phylum Apicomplexa, possess an apical complex comprising several rhoptries and a polar ring. The densely osmiophilic organelles in *T. annulata* sporozoites represent the rhoptries which vary considerably in size, shape and distribution depending on the plane of section. The attachment of sporozoites to target bovine lymphocytes is receptor-dependent, the susceptibility being a function of the receptors. Interiorization of the sporozoites is by invagination of the target cell plasmalemma and is an active process which is temperature-dependent. It is suggested that the base-first orientation of the sporozoite during attachment and interiorization facilitates the early release of the parasite's genomic factors into the host target lymphocyte.

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ULTRASTRUCTURAL CHARACTERISTICS OF IN VITRO PARASITE—LYMPHOCYTE BEHAVIOUR IN INVASIONS WITH *THEILERIA ANNULATA* AND *THEILERIA PARVA*

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ABSTRACT

Jura, W.G.Z.O., Brown, C.G.D. and Rowland, A.C., 1983. Ultrastructural characteristics of in vitro parasite—lymphocyte behaviour in invasions with *Theileria annulata* and *Theileria parva*. *Vet. Parasitol.*, 12: 115—134.

Host—parasite relationships have been studied by electron microscopy using glutaraldehyde—OsO_4-fixed pellets of lymphoid cultures infected in vitro by *Theileria annulata* and *T. parva*. Intracellular presence of the parasite resulted in a progressive and marked lymphoblastoid transformation. The schizont stage periodically provoked the formation of, and adopted an intimate association with, cytoplasmic annulate lamellae in the interphase cell. Annulate lamellae developed from the outer nuclear membrane of the host cell by a delamination process and were taken into the cytoplasmic matrix of the schizont by phagotrophy through the cytostome. Schizont nuclei themselves were seen to divide at the prometaphase stage of host cell mitosis, the division being characterized by the development of intranuclear spindle microtubules anchored in spindle pole bodies. A hypothesis is propounded that *Theileria* parasites, consequent on interiorization, provoke the blastoid transformation and the formation of annulate lamellae through the influence of components of their genomic material on host cell deoxyribonucleic acid (DNA) and that the annulate lamellae represent a species of messenger ribonucleic acid (mRNA) and serve as a monitoring device for the schizont, facilitating the accurate timing of the host cell cyclical events.

INTRODUCTION

Theileria are heterogenetic, heteroxenous, obligately intracellular protozoan parasites which are transmitted by ticks of different genera and which cause disease in a variety of vertebrate animal hosts. In in vitro cultures,

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sporozoites of *Theileria annulata* (Jura, 1981; Jura et al., 1983) and *T. parva* (Stagg et al., 1981) attach to, and invade the target bovine peripheral blood lymphocytes within minutes of incubation. Interiorized sporozoites dedifferentiate into trophozoites within 30 min, feed, increase in size and by an acentric, closed mitosis with intranuclear spindle microtubule development, transform into schizonts around 24 h later (Jura, 1981). The infected host lymphoid cells undergo marked blastoid transformation and repeated replicative cycles for as long as optimal culture conditions are maintained (Malmquist et al., 1970). The indeterminate number of cyclical replications and persistent state of transformation of the infected host cell is dependent on the intracellular residence of the schizont (Malmquist et al., 1970; Moulton et al., 1971) and rapidly terminates with the elimination of the parasite following which the lymphocyte reverts to its normal morphological status (Irvin and Stagg, 1977; McHardy, 1978; Pinder et al., 1981). In established *Theileria*-transformed cultures, a clone of schizont-parasitised lymphocytes is selected by recurrent bipartitioning of schizonts and distribution of daughter schizonts, with accompanying nuclei, into each daughter cell during host cell mitosis (Hulliger et al., 1964); this was demonstrated by ultrastructural studies (Stagg et al., 1980; Musisi et al., 1981; Vickerman and Irvin, 1981). A mutualistic relationship is thus established between the schizont and the host lymphoid cells, the parasite itself an obligate symbiont.

Among the most intriguing problems are: (1) the mechanisms by which *Theileria* schizonts manipulate the metabolic pool of their host cells and (2) the regulatory and monitory devices employed by the parasites to achieve accurate coincidence between its nuclear division and the host cell mitosis.

This study presents ultrastructural features pertaining to the association between the schizont stage of *T. annulata* and *T. parva* and the host lymphoid cells, and a working hypothesis on the mechanism of the symbiotic existence in vitro between *Theileria* parasites and their target cells.

MATERIALS AND METHODS

Establishment of Theileria-*lymphoid cultures*

Details of the methods by which peripheral blood lymphocytes (PBL) were separated from bovine venous blood and by which sporozoites of *T. annulata* and *T. parva* were prepared from infected ticks have been described elsewhere (Jura et al., 1983). In brief, 3-day fed *Hyalomma anatolicum anatolicum* and 4-day fed *Rhipicephalus appendiculatus* ticks infected with *T. annulata* (Hissar) and *T. parva* (Muguga), respectively, were surface sterilized and ground up separately in 3.5% bovine plasma albumin (Armour fraction V, Sigma chemicals) in Eagle's minimum essential medium (Gibco, Europe) to provide ground up tick supernates (GUTS) of concentration equivalent to 4 ticks ml^{-1} and filtered to 8 μ. PBL suspension was prepared from buffy coat obtained from defibrinated blood and layered onto a Ficoll/so-

dium diatrizoate gradient (Ficoll-Paque, Pharmacia Fine Chemicals, Uppsala, Sweden). The cell concentration was adjusted to 8×10^6 cells ml^{-1} of growth medium — RPMI 1640/20% foetal calf serum with 100 i.u. ml^{-1} benzylpenicillin, 100 μg ml^{-1} streptomycin sulphate and 2 mM L-glutamine (all Gibco-Europe).

Cultures were established in 2-cm^2 wells of tissue culture cluster plates (Costar, 205 Broadway, Cambridge, MA, U.S.A.) in which monolayer cultures of a lung fibroblastic cell line, IMR 31, had been established 2—3 days previously. 0.25 ml cell suspension (2×10^6 cells) and 0.25 ml sporozoite suspension were seeded out into each culture well. The cultures were immediately put into a humified plastic box, gassed with 5% CO_2/95% air and incubated at 37°C.

Maintenance of cultures

Each culture well received 0.5 ml growth medium on Day 1 and 1 ml on Day 3. On Day 6, 1 ml cell-free medium was removed from each well and replaced with fresh growth medium. Every 3 days, 1 ml of culture medium was removed and replaced with an equal volume of fresh growth medium. Assessment of cultures for cell morphology and characteristics, and percentage of infected cells (PIC) were undertaken during each medium change. For PIC determinations, 50-μl aliquots of thoroughly mixed cell suspension were cytocentrifuged at 700 rpm for 10 min (Cytospin, Shandon Southern Instruments, Frimley Road, Camberly, Surrey, U.K.) onto microscope slides and smears stained with Giemsa stain. When PIC, determined by counting 200 lymphoid cells, reached 75% cultures were transferred and maintained in disposable plastic flasks of 25 cm^2 surface area (Nunc) according to the technique described by Brown (1979). They were initially passaged at a ratio of 1:1 (i.e., 5 ml cell suspension from the original culture flask transferred to a fresh flask containing 5 ml fresh medium), then passaged at 1:4 and later at 1:10 three times weekly.

Electron microscopy

Samples for electron microscopic examination were obtained at 5, 10, 20, 30, 40 and 60 min, 2, 3, 6, 18, 24 and 48 h of incubation of primary cultures and during passages of established cultures. They were centrifuged at 275 *g* for 10 min, the resultant pellet resuspended in 5 ml serum-free RPMI 1640 and spun at 275 *g* for another 10 min. The cell pellets were processed for transmission electron microscopy as described previously (Jura et al., 1983). They were fixed overnight at 4°C in 2.5% cacodylate-buffered glutaraldehyde, washed several times in cacodylate buffer and post fixed in 1% OsO_4 for 2 h. Post-fixed samples were dehydrated through graded alcohol series, cleared twice in propylene oxide, embedded in araldite and polymerized at 60°C for 2 days. Sections, cut on a Cambridge–Huxley ultramicrotome and stained with uranyl acetate and lead citrate, were examined on a Philips 400 electron microscope.

RESULTS

Normal peripheral blood lymphocytes

Noninfected bovine peripheral blood lymphocytes (Fig. 1) generally manifested some degree of heterogeneity in their ultrastructural characteristics. Common features included (1) a large irregularly shaped or deeply indented vesicular nucleus containing (a) large masses of densely clumped heterochromatin, the chromocentres commonly associated with the inner aspect of the inner nuclear membrane, (b) reticulating, fine heterochromatic threads, the chromonemata, and (c) patches of the much more electron-lucent euchromatin, (d) one or two indistinct, spherular nucleoli associated, in some cells, with small clumps of heterochromatin at the periphery; (2) a generally rudi-

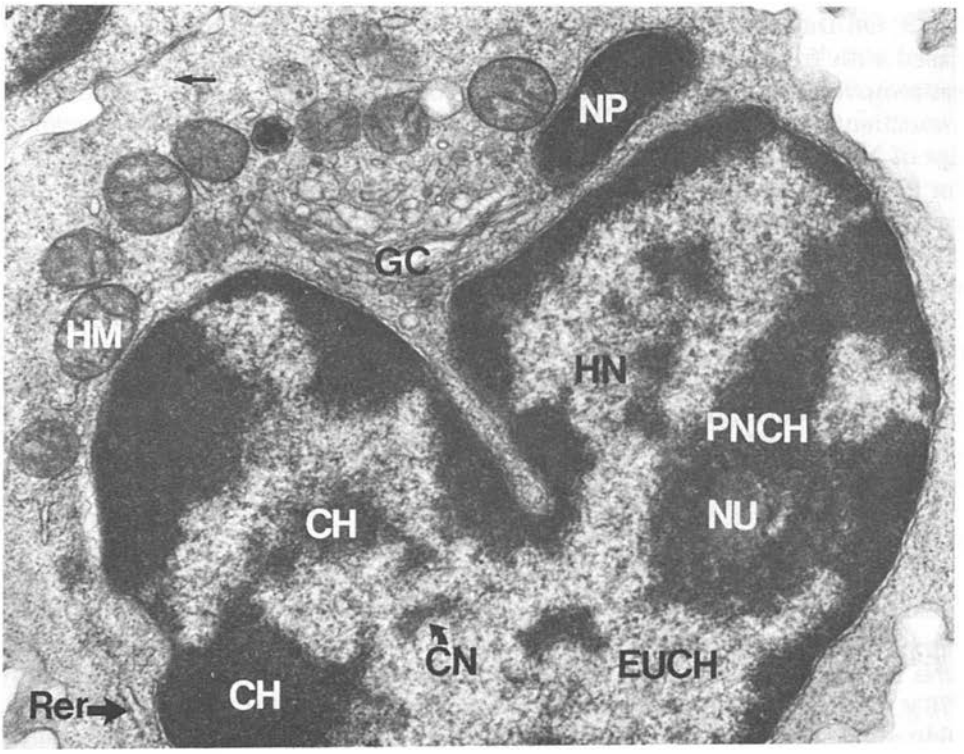


Fig. 1. An uninfected bovine peripheral blood lymphocyte. A deeply indented vesicular nucleus (HN) shows chromocentres (CH) and chromonemata (CN) and patches of electron lucent euchromatin (EUCH). A ring-like nucleolus (NU) is associated with clumps of perinuclear chromatin (PNCH). Golgi complex (GC) and mitochondria (HM) occupy the nuclear "hof" pole of the lymphocyte. Occasional strands of rough endoplasmic reticulum (Rer), and numerous ribosomes (←) are seen in the cytoplasm. A nuclear projection (NP) is an incidental observation. Magnification X 24 018.

mentary, but occasionally well developed Golgi complex; (3) a few well developed cristate mitochondria which showed a tendency to be grouped on the nuclear "hof" pole of the cell; (4) occasional single strands of rough endoplasmic reticulum; (5) numerous free ribosomes; and (6) an inconsistent number and distribution of surface microvilli.

Transforming and transformed Theileria*—lymphoid cultures*

Since similar interaction patterns were manifested by both *Theileria annulata* and *T. parva* in cultures, a general description of the pertinent ultrastructural characteristics is presented. Interiorized sporozoites underwent a dedifferentiation process by losing their rhoptries and polar rings, rounded and transformed into trophozoites (Fig. 2) which gradually increased in size and acquired increasing profiles of rough endoplasmic reticulum and polyribosomes. The parasite rapidly adopted a very close relationship with the host cell nucleus. Each trophozoite eventually transformed into a schizont when its

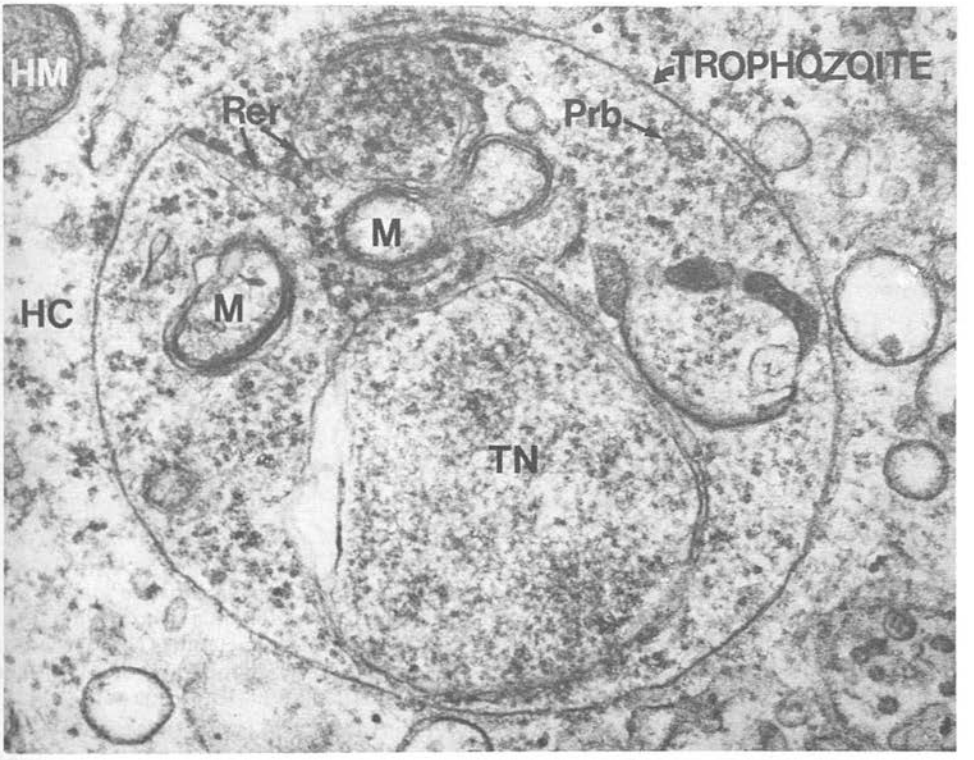


Fig. 2. A fully developed *Theileria* trophozoite within the cytoplasm (HC) of the host lymphocyte. Large numbers of polyribosomes (Prb), well developed profiles of rough endoplasmic reticulum (Rer) and acristate mitochondria (M) are seen in the trophozoite cytoplasm. The nucleus (TN) is non-chromocentric. A host cell mitochondrion (HM) is seen in the top left hand corner of the micrograph. Magnification X 69 738.

nucleus underwent an acentric, closed mitosis with intranuclear spindle development.

Concomitant with the development of the interiorized *Theileria* sporozoites, the infected bovine lymphocytes were observed to undergo well-defined, progressive and marked morphological transformation. The mean cell size increased from 7 μ in the normal lymphocytes to 18—20 μ in the transformed cultures. There was a progressive, centrifugal loosening, dispersion and eventual disappearance of chromocentres and chromonemata so that these heterochromatic regions were replaced by the more metabolically active and lucent euchromatin (Fig. 3). The nucleolus, which in the majority of

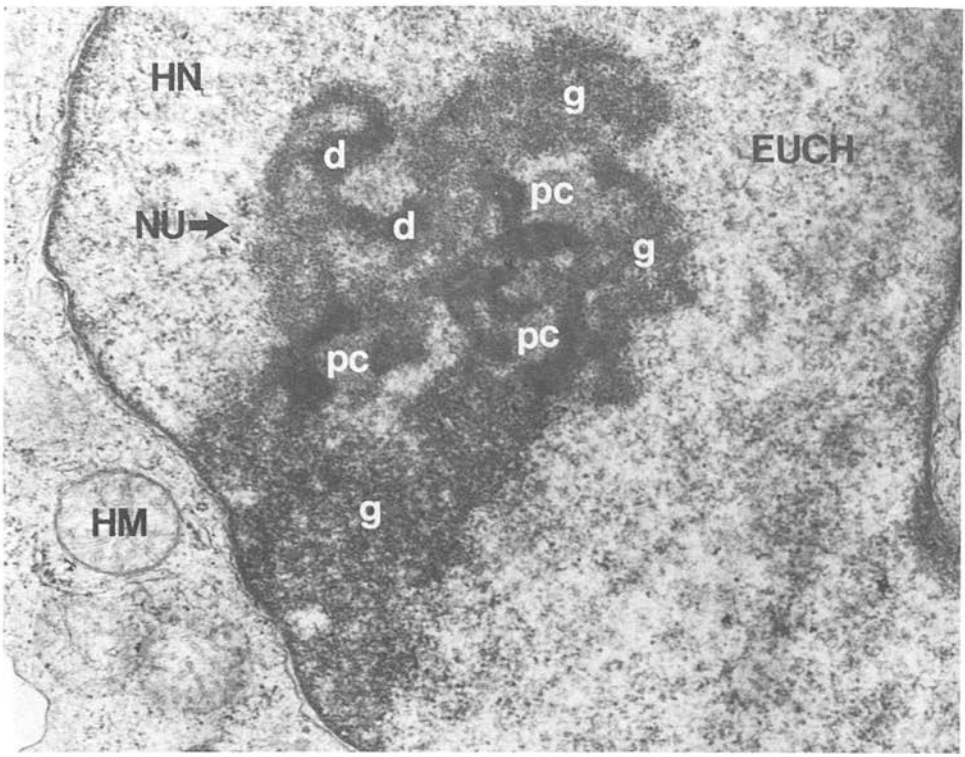


Fig. 3. An electron micrograph of a *Theileria*-transformed lymphoblast showing mainly the nuclear and nucleolar changes. The chromocentres and chromonemata shown in Fig. 1 have loosened and converted to euchromatin (EUCH) while the nucleolus (NU) has undergone elaborate differentiation. Several pale centres (pc) are surrounded by dense (d) and granular (g) components of the nucleolus. Magnification × 24 390.

transformed cells was intimately associated with the inner nuclear membrane, underwent a marked differentiation from its original interphase ring-shape to a reticular configuration composed of several pale centres surrounded by material of varying electron density and granularity. The host cell cytoplasm contained numerous clusters of ribosomes, the polyribosomes, 600–800 Å in diameter (Figs. 7 and 9).

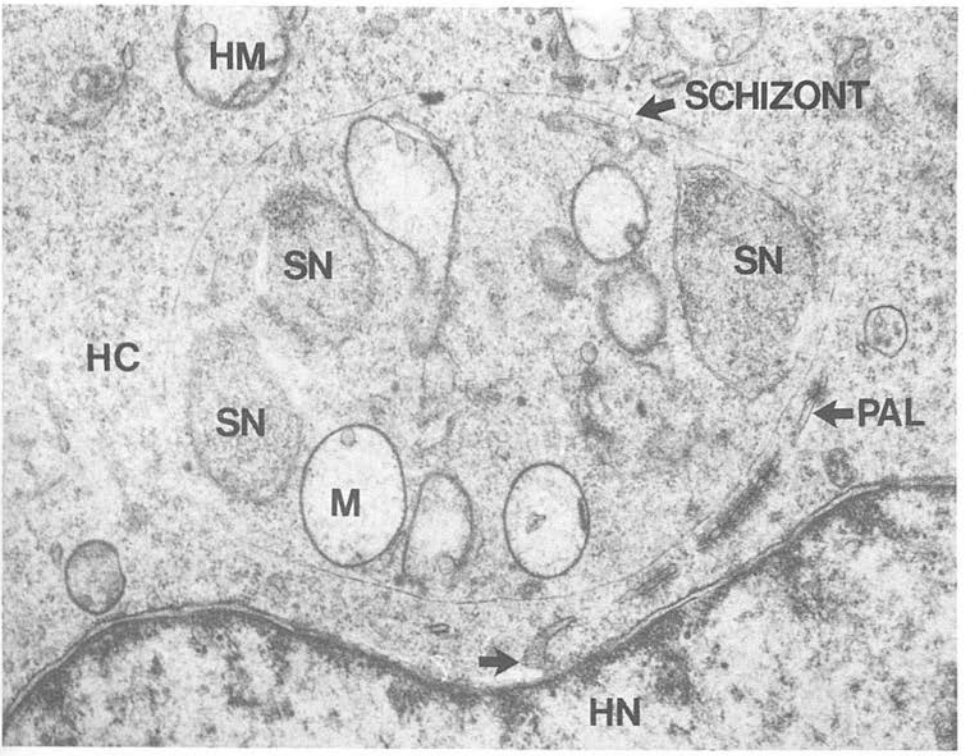
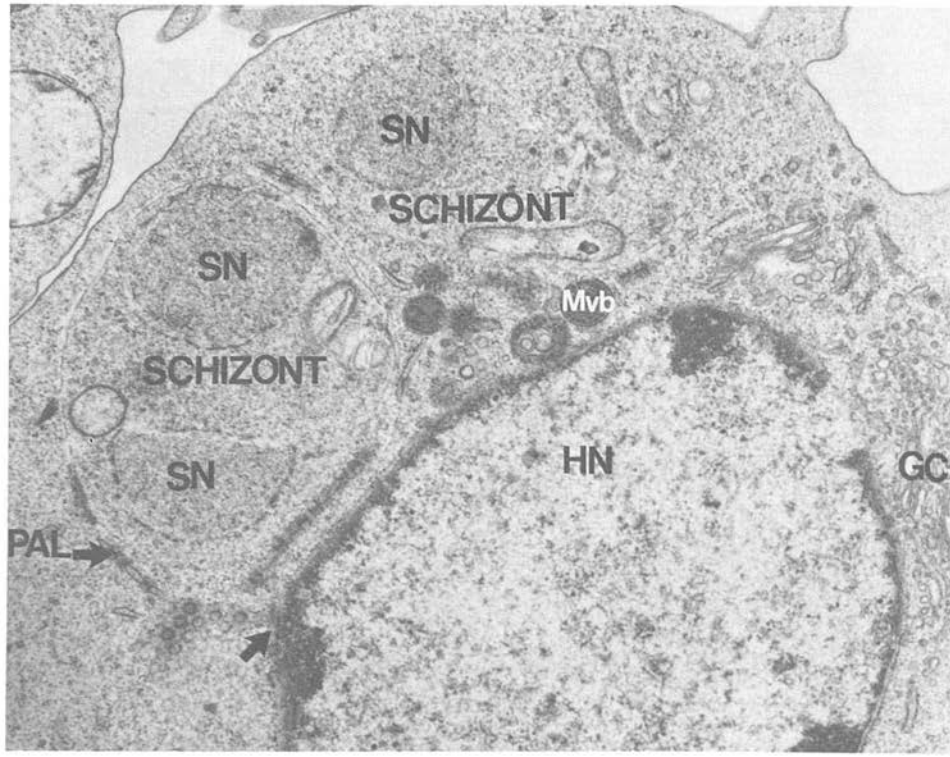
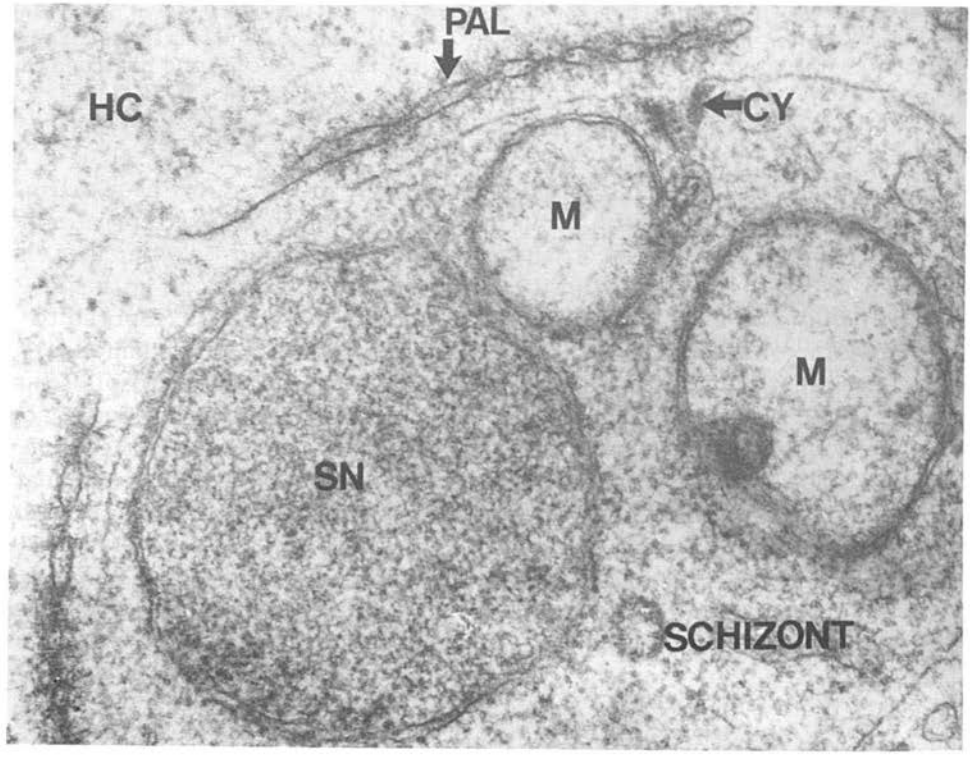


Fig. 4. *Theileria annulata* schizont, shown in the host cell cytoplasm (HC), is lying very close to the host cell nucleus (HN). Porous annulate lamellae (PAL) are shown originating (→) from the outer membrane of the host nucleus by a delamination process and adopting an intimate association with schizont. Several schizont nuclei (SN) and mitochondria (M) are illustrated. Magnification × 25 737.

The schizont stages of *T. annulata* (Fig. 4) and *T. parva* (Fig. 5) were observed tightly associated with porous, parallel cytomembranous type of annulate lamellae (PAL) which are structurally similar to the host cell nuclear envelope. The PAL developed from the outer nuclear membrane in both species of *Theileria* by a delamination process and was observed juxtaposed to the cytostome of the schizont (Fig. 6). Segments of the annulate lamellae were subsequently detected within phagocytic vacuoles in the cytosol of the parasite (Fig. 7). The presence of PAL was transitory. At one time the majority of the schizonts were surrounded by the organelle while at another time this association was not observed. Schizonts where observed to be well clear of the host centriole both in the interphase cell (Fig. 8) and at the onset of prophase (Fig. 9).

In dividing transformed cells in the prometaphase stage of their cycle (Figs. 10 and 11), the schizont nuclei were consistently seen to undergo closed mitosis characterised by a rapid development of intranuclear spindle microtu-


SN
SCHIZONT
SN
Mvb
SCHIZONT
SN
HN
GC
PAL


PAL
HC
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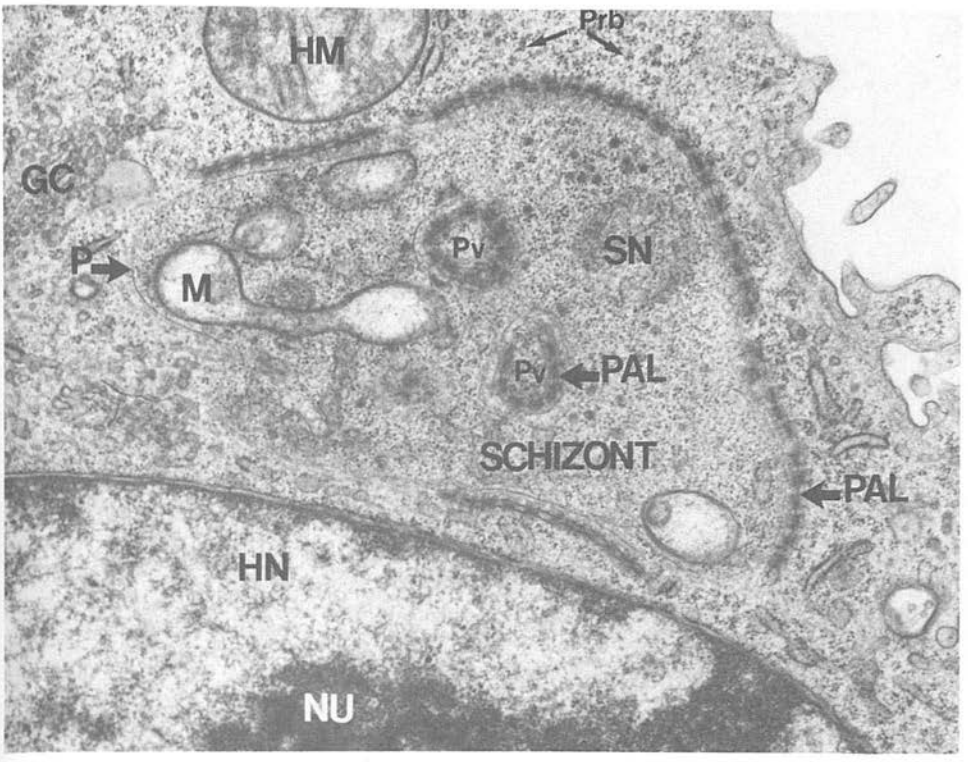

Fig. 7. A section of a *Theileria*-transformed bovine lymphoblast showing a schizont in close proximity to the host nucleus (HN) and with porous annulate lamellae (PAL) tightly applied to its pellicle (P). Fragments of annulate lamellae phagocytosed through the cytostome as shown in Fig. 6 can be seen within phagocytic vacuoles (Pv) in the schizont cytosol. The host cytoplasm is studded with polyribosomes (Prb). Magnification × 25 063.

bules anchored at both ends in spindle pole bodies (Fig. 11). Around prometaphase and later stages of mitosis, host mitotic spindle microtubules developed and inserted on the schizont pellicle. The mitotic spindle microtubules of the dividing host cell were beginning to develop (Fig. 11) and were

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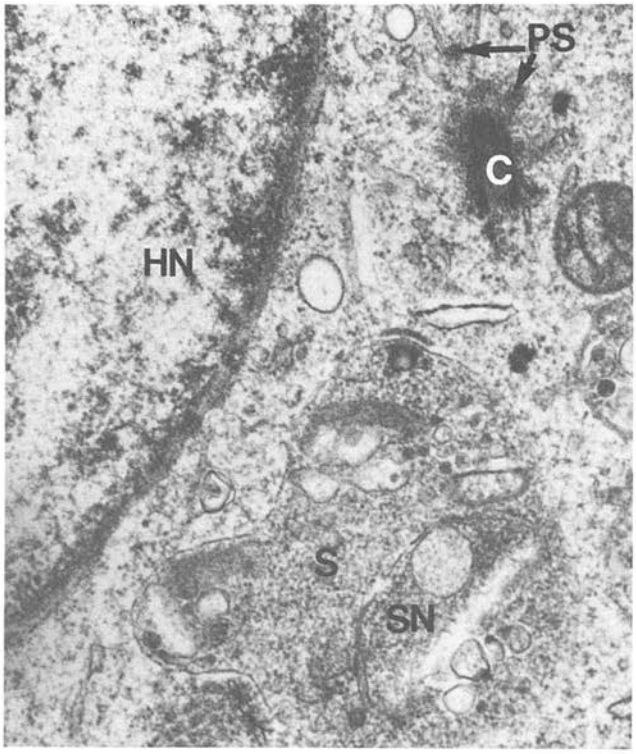
Fig. 5. A bovine lymphocyte infected and transformed in vitro by *Theileria parva*. Two *T. parva* schizonts, lying adjacent to each other in the cytoplasm of the host cell, are in close proximity to the host nucleus (HN). As shown in the case of *T. annulata* (Fig. 4), annulate lamellae (PAL) originate from the outer nuclear membrane (↗) and are tightly associated with the schizonts. Magnification × 18 836.

Fig. 6. An electron micrograph of a section of a *Theileria* schizont with one of its nuclei (SN) and mitochondria (M) lying in the cytoplasm of the host lymphocyte (HC). Porous annulate lamellae (PAL), contiguous to the schizont, are in close apposition to the cytostome (CY) of the parasite and are about to be phagocytosed through the organelle by the schizont. Magnification × 66 130.


PS
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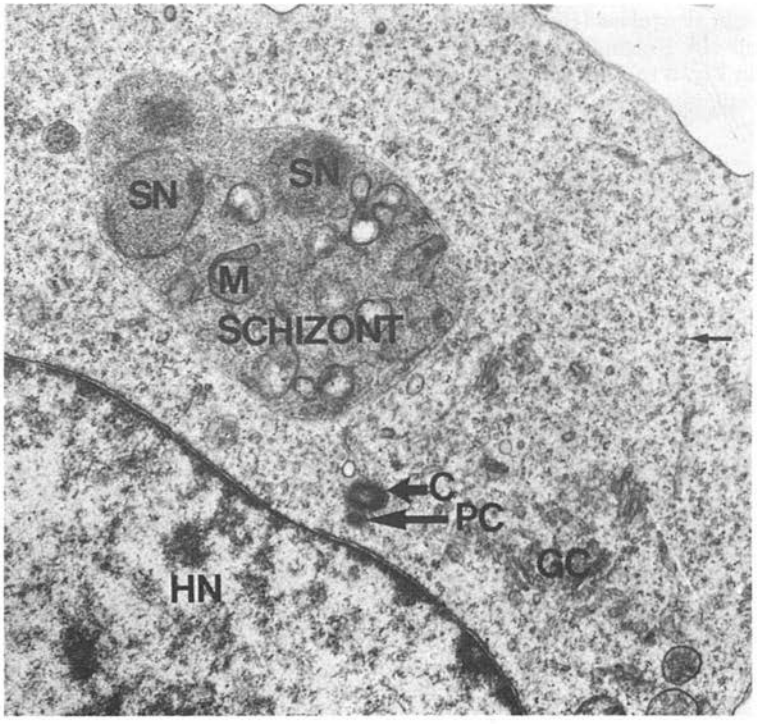
SN
SN
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SCHIZONT
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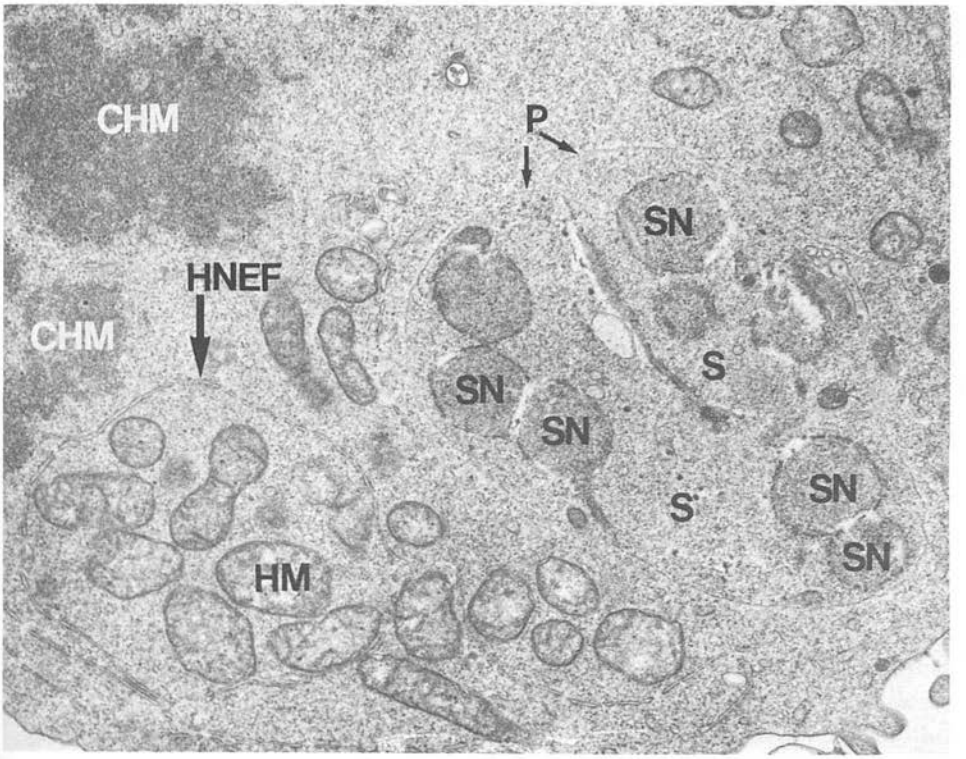

Fig. 10. A *T. parva*-transformed lymphoblast in the prometaphase stage of division showing condensed chromosomal masses (CHM), host nuclear envelope fragments (HNEF), well developed mitochondria (HM) and two schizonts (S) lying with their pellicles (P) adjacent. Several schizont nuclei (SN) are in the process of replication. Magnification × 15 294.

associated with both the host lymphocyte chromosomal masses as well as the schizont pellicle.

DISCUSSION

In this study we demonstrate that infection of bovine peripheral blood lymphocytes with *Theileria* sporozoites results in the manifestation of the

Fig. 8. A section through a *T. parva*-transformed interphase host cell showing a part of a schizont (S) and a centriole (C) associated with pericentriolar satellites (PS). The schizont is devoid of microtubules and still has no association with the centriole together with its satellites and occasional microtubules. Magnification × 30 361.

Fig. 9. A *T. annulata*-transformed lymphoblast just entering prophase as shown by the budding off of a procentriole (PC) from the centriole (C). The schizont, at this stage of host cell cycle, is still devoid of microtubules. A rich compendium of polyribosomes (←) fills the host cytoplasm. Magnification × 15 714.

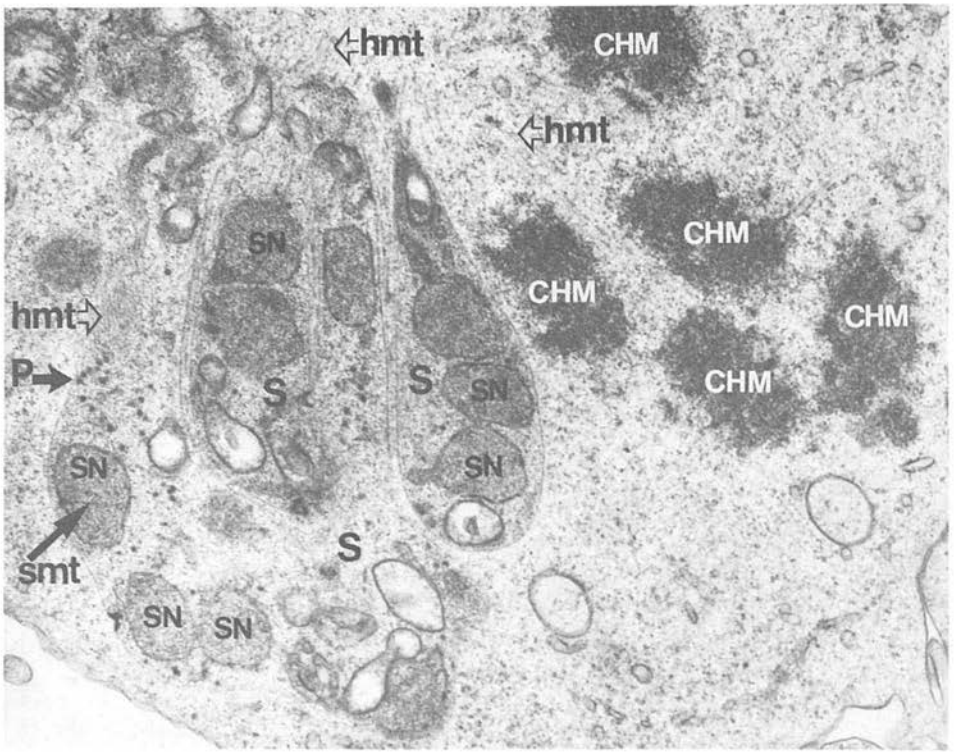

Fig. 11. A *T. annulata*-transformed bovine lymphoblast in the late prometaphase stage showing condensed chromosomal masses (CHM) and three schizonts (S). Several schizont nuclei (SN) are replicating. In one schizont nucleus in the early stages of replication, intranuclear spindle microtubules (smt) can be seen. Host spindle microtubules (Hmt) are beginning to develop and are embedded in the schizont pellicle (P) and host chromosomal masses. Magnification × 15 090.

panoply of characteristic ultrastructural features that define the transformed lymphoblastoid state. The chromocentres and chromonemata, composed of inactive, tightly wound and concentrated chromatin, gradually loosen, disperse and convert to euchromatin, characteristic of nuclei of *Theileria*-transformed lymphoid cultures. Autoradiographic studies on thymocyte nuclei using uridine-H^3 (Allfrey and Mirsky, 1962; Sibatani et al., 1962; Littau et al., 1964) and uridine-2-C^{14} (Frenster et al., 1963) demonstrated that the ribonucleic acid (RNA) precursors are incorporated within euchromatic regions and that the deoxyribonucleic acid (DNA) in these regions actively synthesize RNA which is largely messenger ribonucleic acid (mRNA). The amount of euchromatin in a nucleus reflects the number of genes being transcribed rather than the amount of mRNA produced (Hopkins, 1978). The diffuse distribution of euchromatin observed in the nuclei of *Theileria*-transformed lymphoblasts would thus be construed to implicate the multiplicity of genes participating in the transcription of new mRNAs, and a cor-

respondingly large number of heterogeneous translation products.

Another characteristic structural transformation involves the nucleolus which differentiates into a large reticular structure (Fig. 3) in which the original amorphous, pale centre subdivides into several parts and the perinucleolar heterochromatin changes to an elaborate meshwork of varying density and granularity. The DNA within the nucleolar area contains multiple copies of genes involved in the production and distribution of RNA and is the site of ribosomal ribonucleic acid (rRNA) transcription (Perry, 1969; Threadgold, 1976). It has been shown by electron microscope autoradiography that the transformed lymphocytes readily incorporate uridine-H^3 in nucleolar structure (Winter and Yoffey, 1966; Biberfeld, 1971). The incorporated uridine-H^3 localises on the elaborate meshwork of dense granular material (Vagner-Capodano et al., 1982), an observation which is compatible with the idea that the dense granular components contain newly synthesized RNA (Busch and Smetana, 1970). The extensive differentiation and prominence of the nucleolus in *Theileria*-transformed lymphoid cultures, therefore, signifies a marked increase in nucleolar activity and turnover of ribosomal RNA, implying enhanced protein synthesis.

Concurrent with nuclear and nucleolar changes described in this study is the development of a rich compendium of polyribosomes in the cytoplasm of the host cells. In vivo studies using C^{14} labelled amino acids with subsequent lysis of cells (Warner et al., 1962; De Man and Noorduyn, 1969) have demonstrated that the site of protein synthesis is not the single ribosome, but rather the multi-ribosome–mRNA complexes, the polyribosomes, since it is these complexes which are capable of incorporating C^{14} labelled amino acids into protein. In the complexes the ribosomes act as the co-ordinating unit relating mRNA and tRNA while the strand of mRNA which holds the ribosomes together contains the information for assembling the tRNA-amino acid couple to the polypeptide chain. There is, therefore, no doubt from the above discussion that the infection of lymphoid cells with theilerial parasites induces a complex, interrelated chain of reactions at molecular level that underly the characteristic lymphoblastoid or transformed state.

Annulate lamellae (Swift, 1956) have been most commonly described in rapidly differentiating cells such as oocytes, spermatocytes, embryonic as well as tumour and cancer cells (Kessel, 1968, 1973; Wischnitzer, 1970; Franke, 1974; Maul, 1977). Their occurrence, but not their origin, in *Theileria*—lymphoid cultures has been documented (Musisi et al., 1981; Vickerman and Irvin, 1981). Direct evidence that they originate from the nuclear envelope was first provided in studies on the developing *Necturus* oocyte (Kessel, 1963) and subsequently verified in developing echinoderm oocytes (Kessel, 1964) and tunicate oocytes (Kessel, 1965). Material associated with annulate lamellae in the dragon-fly oocyte (Kessel and Beams, 1969), *Drosophila melanogaster* spermatocytes (Kessel, 1981) and sea urchin eggs (Conway, 1971) has been shown by cytochemical methods to contain RNA. Incorporation of uracil-H^3 in annulate lamellae in *Necturus* oocyte has been reported (Kes-

sel, 1968). Although numerous studies have been undertaken on annulate lamellae, their functional significance remains speculative. It has been suggested that they: (1) carry nuclear-derived information to the cytoplasm (Moses, 1964; Swift, 1958); (2) are involved in the assembly of stored gene products and activation of ribosomal subunit material into functional polyribosomes (Kessel, 1981); and (3) contribute towards the formation of cytoplasmic microtubule complex (Chemnitz and Salmberg, 1978; Musisi et al., 1981). It has been observed that annulate lamellae appear not only transitorily or cyclically (Wischnitzer, 1970; Maul, 1977), but also that they are present only at specific times. For example, in maturing human spermatids, annulate lamellae are observed at stages designated Sb1 and Sd1 (Smith and Berlin, 1977), while in developing chick myocardial cells, they are present only on the eleventh day and not on the seventh or eighth day of incubation (Merkow and Leighton, 1966). The observations that annulate lamellae originate from the nuclear envelope, are associated with RNA and appear at specific stages in developmental events would appear to imply that they contain coded instructions necessary either for initiating, regulating or monitoring certain cellular activities.

The nuclear, nucleolar and polyribosomal changes we have described in the present study, coupled especially with the host nuclear annulate lamellae- -schizont interactions together with the precise timing of schizont nuclear replication are of considerable interest and provoke some very challenging questions about the molecular mechanisms underlying *Theileria*-induced host lymphoid cell transformation, and the functional significance of annulate lamellae in *Theileria*-transformed lymphoid cell lines.

In a previous paper (Jura et al., 1983), it was suggested that *T. annulata* sporozoites, during their invasion of target lymphocytes, release components of their genomic material into the host cell. These would reach the host cell nucleus, recognize and bind to specific gene sites to initiate the transcription of mRNA molecules. The cytoplasm to nucleus transfer may be achieved, as in the case of steroid hormones (Green, 1980), by complexing with specific receptor proteins in the target cell cytoplasm thus increasing their affinity for chromatin and hence the migration to the nucleus. The transcriptional events may be spread over a number of phases so that during the first 24–48 h of infection, for example, the majority of mRNA species produced code for trophozoite—host lymphocyte interactions. The second phase species would be responsible largely for the stable, perpetual and symbiotic co-existence characteristic of *Theileria*-lymphoid cultures in vitro, while especially in vivo the late transcription products may be concerned with the terminal stages of the schizont, and the accurately timed switch to piroplasm production (Jarrett et al., 1969; Jura and Losos, 1980). The incorporated *Theileria* genomic components thus represent a self-contained transcriptional unit which utilizes host cell replicative machinery yet remains subject to influences originating from outwith itself. All *Theileria* species so far grown in vitro, for example *Theileria parva* (Malmquist et al., 1970), *T. annulata* (Hul-

liger, 1965), *T. lawrencei* (Stagg et al., 1974), *T. hirci* (Hooshmand-Rad and Hawa, 1975) and *T. taurotragi* (Stagg et al., 1976) induce host lymphoid cells to undergo unimpeded replication. It has also been shown that when *T. parva*-transformed bovine lymphoblastoid cells are inoculated subcutaneously into irradiated athymic (nude) mice, tumour-like masses composed of bovine cells develop at the site of inoculation (Irvin, 1977; Irvin et al., 1977) followed by progressive growth with extensive infiltration of parasitized cells to all organs, especially the lungs. A careful ultrastructural examination of *Theileria*-transformed cell lines in this investigation has not demonstrated any virus particles. Elimination of the intracellular schizonts by drugs (McHardy, 1978; Pinder et al., 1981) terminates the blastoid transformation characteristics so that the host cell reverts to its normal morphological status, thus pinpointing *Theileria* as the agent responsible for the observed changes. The insinuation of parasite genome into the host cell nucleus has also been postulated in *Xenoma* production by the microsporidian, *Glugea weissenbergi* in the stickleback *Apeltes quadracus* (Sprague and Vernick, 1968). In this case the infective sporoplasms of *Glugea* are believed to be introduced directly into the host cell nucleus where as a result of the interaction between the parasite and the host cell DNAs, symbiotic complexes of multinucleate host cell and intracellular parasites, the *Xenoma* (Weissenberg, 1968) are formed. A similar relationship has been described in the turbot *Scophthalmus maximus* (Ferguson and Roberts, 1976) in which every cell of a lymphoma-like condition was parasitized by protozoan parasites.

We believe that annulate lamellae are a transcription product representing a species of mRNA and function in communicating cyclical changes in host cell nucleus to *Theileria* schizonts and that through their degradation and assimilation (Fig. 7), the schizont is able to monitor the host cell chromosomal changes and replicate its nuclei in the prometaphase stage as we have shown in this study (Figs. 10 and 11). The nuclear replication is timed such that when host cell anaphase chromosomal migration starts, there is concurrent distribution of schizont nuclei into each daughter cell in a similar manner as for host chromosomes through the instrument of the host mitotic spindles (Fig. 11). Where host spindle microtubule activity is interfered with, as for example, by treatment with colchicine (Hulliger et al., 1964) or where the host mitotic process is disturbed by ionizing irradiation (Irvin et al., 1975), the schizont assesses the host nuclear activity correctly and replicates its nuclei so that up to a hundred schizont nuclei may be found per cell. Whereas colchicine impairs microtubule polymerization (Borisy and Taylor, 1967) and cells are arrested in the metaphase stage, X-irradiation of cultures blocks cells in the G2 phase from entering into mitosis due to chromosomal breakage (Das, 1963) and increases mitotic index even up to 80% (Padilla et al., 1966). The schizont is more resistant than the host cell to both treatments and develops normally while the host cell replication is severely affected.

Musisi et al. (1981) believed the annulate lamellae originated from the Golgi complex. We have, however, demonstrated clearly in this study (Figs. 4

and 5) that the organelle originates from the outer nuclear envelope and that the origin has no bearing to the Golgi complex. Our observations that the interphase (Fig. 8) and replicating (Fig. 9) centrioles have no connection with the schizont and that the host mitotic spindle microtubules insert on the schizont pellicle (Fig. 11) at the same time as they attach to the host chromosomal masses, are at variance with those of Vickerman (1980) who reported that during centriole replication the schizonts become encased in host cell microtubules and that the fibres do not insert on the membrane of the schizont, but merely encase the parasite. After examining numerous samples of *Theileria*-transformed lymphocytes we have no doubt that the schizont–microtubule associations which have been described in the interphase cells (Stagg et al., 1980; Musisi et al., 1981) represent newly formed daughter cells in which residual mitotic spindle microtubules still maintain their associations with the daughter schizonts. Whereas Hulliger et al. (1964), Musisi et al. (1981) and Vickerman and Irvin (1981) observed that the cleavage of the schizont and distribution of daughter schizonts with accompanying nuclei to daughter cells is synchronized with host cell mitosis, we have demonstrated in this study that the schizont nuclei themselves replicate earlier during the prometaphase stage of the host cell cycle just before the bipartitioning and distribution of schizonts to daughter cells.

CONCLUSIONS

Blastoid transformation of target host lymphoid cells and obligate symbiotic co-existence in vitro were achieved by *Theileria* parasites through the influence of their genetic factors on host cell DNA. The nuclear envelope-derived annulate lamellae are believed to be a transcription product representing a species of mRNA and function in communicating cyclical changes in the host cell nucleus to *Theileria* schizonts so that through their degradation and assimilation, schizonts are able to monitor host cell chromosomal changes.

Theileria schizonts replicate their nuclei during the prometaphase stage of host lymphocyte cycle while the distribution of daughter schizonts with accompanying nuclei to daughter cells is synchronized with host anaphase chromosomal migration so that if this migratory process was inhibited or delayed, the number of schizont nuclei per cell increased disproportionately.

Host spindle microtubules insert on schizont pellicles and host chromosomal masses during metaphase and were responsible for the cleavage by traction of schizonts and distribution of daughter schizonts with accompanying nuclei to daughter cells. Schizont–microtubule associations were neither observed during interphase nor early prophase stages of *Theileria*-transformed cells.

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